

Vol. III of IV (Appx12136-19916)
Nos. 24-1324, 24-1409

**UNITED STATES COURT OF APPEALS
FOR THE FEDERAL CIRCUIT**

NATERA, INC.,

Plaintiff-Appellee,

v.

NEOGENOMICS LABORATORIES, INC.,

Defendant-Appellant.

Appeals from the United States District Court for the Middle District of North
Carolina, No. 1:23-cv-00629; Hon. Catherine C. Eagles

NON-CONFIDENTIAL JOINT APPENDIX

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NOTE: Documents filed under seal in the district court used both green and yellow highlighting to indicate confidentiality. But the yellow highlighting at Appx7624, Appx7926-7929, Appx10490-10492, and Appx12169-12170 has been unsealed in the district court and does not indicate confidential information; at Appx7624 and Appx7926-7929 only the green highlighting indicates confidentiality. The slides at Appx21068-21319 also includes non-confidential markings; only the full-page yellow rectangular markings indicate relevant confidentiality. The redaction appearing at Appx20714 was part of the underlying document as originally filed in the district court. Appx12169 of the non-confidential addendum contains a paragraph number that is highlighted but not redacted; the highlighting does not indicate confidentiality.

CONFIDENTIAL MATERIAL OMITTED

The non-confidential version of this appendix redacts material filed under seal pursuant to the protective order issued by the district court. As required by Federal Circuit Rule 25.1(e)(1)(B), the table below notes the specific pages with redacted material in the non-confidential appendix and the general nature of that material.

Description of Redacted Material in Non-Confidential Appendix

Document	Pages	Description
Metzker Declaration (Dkt. 17)	Appx7624	Natera's confidential product information
Moshkevich Declaration (Dkt. 18)	Appx7926-7929	Natera's confidential product information
NeoGenomics' Response in Opposition to Motion for Preliminary Injunction (Dkt. 107)	Appx10480-104481, Appx10485, Appx10487, Appx10494-10495, Appx10497-10500, Appx10502-10504	NeoGenomics' confidential financial, technical, and customer information
Excerpts from Moshkevich Deposition (Dkt. 108-6)	Appx10774-10787	The material omitted from page Appx10777 includes information relating to Natera's internal business structure. The material omitted from pages Appx10778-79 includes information relating to Natera's internal market analyses and marketing strategy. The material omitted from pages Appx10780-83 and Appx10785 includes information relating to Natera's business development efforts and customer relationships. The material omitted from pages Appx10786-87 includes information relating to Natera's IP licensing disputes and business relationships.

Sikri Declaration (Dkt. 94)	Appx11274-11275, Appx11277-11284, Appx11286-11294	NeoGenomics' confidential financial, technical, and customer information
Van Ness Declaration (Dkt. 97)	Appx12171	Natera's confidential business relationships
Metzker Declaration (Dkt. 141)	Appx18746 Appx18898-18901	NeoGenomics' confidential technical information
Natera's Reply in Support of Motion for Preliminary Injunction (Dkt. 144)	Appx19187, Appx19191, Appx19194-19195, Appx19198, Appx19200-19204	The material omitted at Appx19187, Appx19191, and Appx19194-19195 includes NeoGenomics' confidential product information. The material omitted from page Appx19198 includes a summary of a confidential communication regarding inventorship of the '454 patent. The material omitted from pages Appx19200-19204 includes information relating to Natera's customer relationships and NeoGenomics' clinical research.
Deposition of Malackowski (Dkt. 144-5)	Appx19518	The material omitted from page Appx19518 includes information relating to NeoGenomics' market opportunities.
Sikri Declaration (Dkt. 183-1)	Appx20805-20806 Appx20809	NeoGenomics' confidential client financial and customer information
NeoGenomics' Reply in Support of Motion to Stay Preliminary Injunction (Dkt. 196)	Appx20933	NeoGenomics' confidential financial information
NeoGenomics' Preliminary Injunction Presentation	Appx21251 Appx21269-21296 Appx21313 Appx21316-21318	Natera's confidential technical and business information

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342. It is my opinion that the Asserted Claims of the '035 patent are “methods-of-detection” claims. Claim 1 of the '035 patent recites methods for detecting the presence of SNPs (DNA code variants) “associated with cancer.” The methods disclosed in asserted claim 1 of the '035 patent include the following steps:

- a. tagging isolated cfDNA;
- b. amplifying the tagged cfDNA, including targeted amplification of a plurality of single nucleotide polymorphism (SNP); and
- c. sequencing the plurality of SNP loci on the cfDNA.

343. The Asserted Claims of the '035 patent detect or measure the amount of certain genetic data (SNPs associated with cancer)—in other words, the Asserted Claims detect a natural phenomenon. Natera did not invent or discover cfDNA associated with cancer, the relationship between cfDNA and cancer, or the correlation cfDNA has with cancer. Further, the disclosures of the '035 patent are generic and fail to provide guidance as to which SNPs are associated with any particular type of cancer or cancer in general, or even what SNPs are associated with healthy cells. The user is left to figure this information out for themselves.

344. The prosecution and allowance of the '035 patent confirm that the Asserted Claims are directed to generic detection of DNA code associated with cancer. In response to a rejection for obviousness, the Applicant amended the claims to recite sequencing SNP loci comprising 25-2000 loci “associated with cancer.” Ex. D.I. 53-1, Ex. D.I. 53-2 at 2. Further, the Applicant claimed that the prior art Chuu reference “fails to teach or suggest targeted amplification of 25-2,000 target loci associated with cancer in a single reaction volume.” Ex. D.I. 53-2 at 5. Following this amendment and argument, the claims of the '035 patent were allowed by the Examiner, who explained that the amendment to add the “associated with cancer” limitation, to the exclusion of

testing fetal cfDNA, led to allowance. Ex. D.I. 52-3 at 3. In my opinion, this confirms that the '035 patent is directed to a natural phenomenon.

345. Additionally, I understand that Natera and its inventors have repeatedly asserted that detecting tumor-derived cfDNA is an “equivalent condition” to detecting fetus-derived cfDNA. D.I. 53-3 ¶ 10 (Natera inventor arguing in affidavit that “it should be noted that cfDNA from a fetus in the plasma or serum is considered by a person of ordinary skill in the art as an ‘equivalent condition’ to the presence of ctDNA in the relevant art.”), Ex. D.I. 53-4 at 5 (Natera arguing during patent prosecution that “the same lab techniques including extraction of cell-free DNA, amplification of targeted loci, and sequencing of the amplicons can be used in both the fetal DNA context and cancer DNA context.”); *see also* Ex. D.I. 53-5 at 1, 3, 6. Even if detecting SNPs associated with cancer was patent eligible, Natera has repeatedly argued that this is the same concept as detecting variants from a fetus, which Natera has also argued cannot be an inventive concept.

346. I also understand that Natera previously argued that (1) cfDNA occurs naturally as a result of cell death in a transplant recipient’s body; and (2) correlating the detected quantity of donor-specific cfDNA to transplant health, are both natural phenomenon that are not patent eligible. Ex. 10 at 14 (arguing that “[t]he Claims are directed to detecting a natural phenomenon—donor-specific cfDNA from a transplanted organ that circulates in the blood of a transplant recipient. The '652 patent claims are directed to the additional unpatentable concept of correlating the detected quantity of donor-specific cfDNA to transplant health—another natural phenomenon”). I have reviewed the claims of the '652 patent, and it is my opinion that the “detecting” steps recited by the Asserted Claims of the '035 patent are broader, more generic, and recite less technical detail than the “determining” or “quantifying” steps of the '652 patent.

Therefore, to the extent the '652 patent is characterized as being directed to detecting natural phenomenon, the Asserted Claims of the '035 patent are even more so.

347. Therefore, the Asserted Claims of the '035 patent are directed to detecting a natural phenomenon, and are hence, patent ineligible under §101.

2. The '035 Patent, Natera's Expert Witnesses, and Natera's Own Admissions State That the Techniques Recited by the Asserted Claim Are Standard, Routine, and Conventional

348. In my opinion, the claims of the '035 patent identify the recited steps only at a high level of generalization. The claims do not identify any particular approach for performing the steps. The written description states how different ways of carrying out the techniques recited in those steps, and the combinations of them, are conventional, common, or already in use.

349. Neither the '035 patent's written description nor the claims identify anything as being nonconventional or innovative about the limitations recited in the claims.

350. The '035 patent incorporates by reference a 2008 publication by Varley et al., which demonstrates that the claims were known in the art well in advance of the claimed inventions. D.I. 52-4 at 93:41-58 (citing D.I. 52-5), 250:9-11; D.I. 52-5 at Abstract, 1845.

a. The '035 Patent and Natera's Expert Witness State that Tagging DNA Was a Well-Understood, Routine, and Conventional Technique by 2011

351. Claim 1 of the '035 patent recites "tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products." D.I. 1-2 at claim 1. Tagging DNA was well known to a POSA.

352. Nowhere does the '035 patent state that the methods for tagging described in the patent specification involve anything new, innovative, or novel. To the contrary, the specification

of the '035 patent makes clear that DNA tagging and part of amplification and sequencing was known in the art:

As is known in the art, the term “sequence tag” refers to a relatively short (e.g., 15-100) nucleic acid sequence that can be used to identify a certain larger sequence, e.g., be mapped to a chromosome or genomic region or gene.

D.I. 1-2 at 187:16-20.

353. Likewise, the '035 patent cites to prior art publications describing routine, standard, tagging techniques known in the art, including barcoding (all emphases below are added):

'035 patent at 9:

- Craig, D. W. et al., “Identification of genetic variants using *barcoded multiplexed sequencing*”, Nature Methods, vol. 5, Oct. 2008, 887-893.

'035 patent at 13:

- McCloskey, M. L. et al., “Encoding PCR Products with *Batchstamps and Barcodes*”, Biochem Genet., vol. 45, Oct. 23, 2007, 761-767.

'035 patent at 14:

- Miner, B. E. et al., “Molecular *barcodes* detect redundancy and contamination in hairpin-bisulfite PCR”, Nucleic Acids Research, vol. 32, No. 17, Sep. 30, 2004, 1-4.

'035 patent at 19:

- Parameswaran, P., et al., “A pyrosequencing-tailored nucleotide *barcode* design unveils opportunities for large-scale sample multiplexing”, Nucleic Acids Research, Oct. 11, 2007, 9 pages.

354. Further, Natera’s expert, Dr. Michael Metzker, has repeatedly acknowledged that tagging was well known in the art by 2011:

Solexa’s next generation sequencer was released in late 2006 and achieved widespread commercial use by 2007. Barcoding or sample tagging was equally prevalent.

Ex. 12 ¶ 115; *see also* Ex. 6 at 114:24-115:1 (“Ligation would be -- or PCR -- the two most common ways of [tagging cfDNA].”); Ex. 37 (“It has been well known for many years that barcodes can be added to target nucleic acid sequences either using PCR primers or using ligated adaptors”).

Next generation sequencers could sequence millions of DNA molecules in a single run and thereby made feasible sequencing DNA from different individuals at the same time. To distinguish samples from different individuals, DNA from a particular individual was “tagged” or “indexed” such that each individual sample had a separate tag, index or barcode. Thus, a “tagged” or “indexed” library of that individual’s DNA fragments could be created and the “tag” or “index” could be used to associate a particular DNA with an individual. The advantage of “indexing” was that DNA from different individuals could be pooled and sequenced together in a single reaction.

Ex. 12 ¶ 55.

[The 2010 Fluidigm reference] states: “[i]f desired, *tagged target nucleotide sequences generated as described herein* may be analyzed by *DNA sequencing*.”

Id. ¶ 94. Further, Dr. Metzker has opined that the “use of indexed DNA library for multiplexed next generation sequencing was widespread in the art,” and that the use of an “indexed DNA library” is the same as the use of tags. *Id.* at 38, ¶ 59 (“In addition to the term barcode, these molecular codes have been referred to as tags, multiplex identifiers, and indexes”). In a case before the Patent Trial and Appeal Board, Dr. Metzker provided an expert declaration on behalf of Natera in which he provided nine paragraphs of examples of the use of barcoding in the art:

- He cites to McKenna, who used barcoded primers for sequencing. These barcoded primers had a structure of Sequencing Primer-Barcode-Targeted primer, and serve the function to uniquely tag a given sample. *Id.* ¶¶ 60-61, 65.
- As another example, Dr. Metzker describes the Illumina GA sequencer, which he described as “the most commonly used next-generation sequencer” as of 2010. The first step of the workflow of the Illumina GA sequencer was DNA library preparation, which would have included barcoding. *Id.* ¶ 62.
- As yet another example, Dr. Metzker describes Bentley, which ligated adaptors to sheared DNA fragments for amplification and sequencing. *Id.* ¶ 63.
- As yet another example, Dr. Metzker cited to Quail, which used the Illumina GA system with barcoded primers to analyze DNA fragments and generate an indexed library. *Id.* ¶ 64.
- As yet another example, Dr. Metzker describes Craig, which provided a generalized framework for multiplexed resequencing of targeted genome regions. In the described protocol, “a unique indexed-adaptor sequences [*sic*] was ligated” to DNA fragments. *Id.* ¶ 67.

355. In addition to the Illumina GA sequencer system from 2010, Dr. Metzker described even earlier systems wherein barcoding was widespread and used in the art:

As discussed above, in the relevant timeframe, next-generation sequencing was in widespread use. In fact, 454 Life Sciences Corporation's sequencer, known as Genome Sequencer 20, was commercially in use by 2005. Likewise, Solexa's next generation sequencer was released in late 2006 and achieved widespread commercial use by 2007. *Barcoding or sample tagging was equally prevalent.*

Id. ¶ 115.

356. As yet another example, as early as 2009, it was known that a universal PCR could be used with primers that included sequencing oligos and barcodes, such as what was taught by Varley and Mitra. D.I. 52-5 at 1849 ("The universal PCR used primers tailed with *454 Life Sciences A or B oligo at the 5' end, followed by a sample-specific DNA sequence* and ending at the 3' end with the same universal primer sequence ligated to the amplicons in the Nested Patch PCR procedure.").

357. In sum, the '035 patent and Natera's expert witness show that the technique of tagging cfDNA as claimed in the '035 patent was well-known, routine, and conventional in 2011.

b. The '035 Patent, Natera's Expert Witnesses, and Natera's Own Admissions State That DNA Amplification Was a Well-Understood, Routine, and Conventional Technique by 2011

358. Claim 1 of the '035 patent recites methods for "amplifying the tagged products one or more times to generate final amplification products." D.I. 1-2 at claim 1. DNA amplification, including PCR, was well known to a POSA.

359. The written description of the '035 patent includes the following exemplary disclosures, describing various amplification techniques, including PCR, that it characterizes as routine, and available at the time of the filing to perform the claimed methods:

Performing a highly multiplexed PCR amplification using methods known in the art results in the generation of primer dimer products that are in excess of the

desired amplification products and not suitable for sequencing.

Id. at 86:10-13 (emphasis added).

In some embodiments, the preparation of the DNA may involve ***amplification***, separation, purification by chromatography, liquid separation, isolation, preferential enrichment, preferential amplification, targeted amplification, ***or any of a number of other techniques either known in the art*** or described herein.

Id. at 131:32-37 (emphasis added).

If desired, any of the PCR conditions disclosed herein or 35 ***any standard PCR conditions can be used*** to test a primer library to determine, e.g., the percent of primer dimers, percent of target amplicons, and percent of target loci that are amplified. If desired, standard methods can be used to optimize the reaction conditions to improve the performance of a primer library.

Id. at 125:35-41 (emphasis added).

[S]imultaneous amplification of many target nucleic acids in a sample of interest can be carried out by combining many oligonucleotide primers with the sample and then subjecting the sample to polymerase chain reaction (PCR) conditions in a process known in the art as multiplex PCR.

Id. at 2:66-3:4.

Some embodiments of the present disclosure involve the use of “Linked Inverted Probes” (LIPs), which have been previously described in the literature, to amplify the target loci before or after amplification using primers that are not LIPs in the multiplex PCR methods of the invention.

Id. at 111:11-15.

In some embodiments, the PCR products are sequenced as described 60 in Example 15 or using standard sequencing methods.

Id. at 129:59-61.

There are also sequencing methods, for example the ILLUMINA SO LEXA GENOME SEQUENCER or the ABI SOLID GENOME SEQUENCER, wherein the genetic sequence of fragments of DNA are sequenced; upon extension of the strand of DNA complementary to the strand being sequenced, the identity of the extended nucleotide is typically detected via a fluorescent or radio tag appended to the complementary nucleotide.

Id. at 184:26-34.

The following protocol was used for 800-plex amplification of DNA isolated from maternal plasma from a euploid pregnancy and also genomic DNA from a triploidy 21 cell line using standard PCR (meaning no nesting was used).

Id. at 210:26-34.

An aliquot of the STA products was then amplified by standard PCR for 10 cycles with 1 uM of tag-specific forward and barcoded reverse primers to generate barcoded sequencing libraries.

Id. at 214:33-36.

An aliquot of the STA products was then amplified by standard PCR for 15 cycles with 1 uM of tag-specific forward and barcoded reverse primers to generate barcoded 45 sequencing libraries.

Id. at 214:41-43.

An aliquot of the STAR 2 products was then amplified by standard PCR for 12 cycles with 1 uM of tag-specific forward and barcoded reverse primers to generate barcoded sequencing libraries.

Id. at 218:12-16.

360. Likewise, the '035 patent cites to a wealth of prior art publications describing routine, standard, amplification techniques known in the art, including PCR (all emphases below are added):

'035 patent at 7:

- Binladen, J. et al., "The Use of Coded **PCR** Primers Enables High-Throughput Sequencing of Multiple Homolog **Amplification** Products by 454 Parallel Sequencing", PLOS One, Issue 2, Feb. 2007, 9 pages.
- Siebert, P. D. et al., "An improved **PCR** method for walking in uncloned genomic DNA", Nucleic Acids Research, vol. 23, No. 6, 1995, 1087-1088.
- Tewhey, R. et al., "Microdroplet-based **PCR enrichment** for largescale targeted sequencing", Nature Biotechnology, vol. 27, No. 11, Nov. 2009, 1025-1031.

'035 patent at 9:

- Cheung, V. G. et al., "**Whole genome amplification** using a degenerate oligonucleotide primer allows hundreds of genotypes to be performed on less than

one nanogram of genomic DNA”, Proceedings of the National Academy of Sciences, USA, vol. 93, Dec. 1996, 14676-14679.

'035 patent at 10:

- D'Aquila, Richard et al., “Maximizing sensitivity and specificity of **PCR** by pre-**amplification** heating”, Nucleic Acids Research, 19(13), 1991, p. 3749.
- Dietmaier, W. et al., “Multiple Mutation Analyses in Single Tumor Cells with Improved **Whole Genome Amplification**”, American Journal of Pathology, vol. 154, No. 1, Jan. 1999, 83-95.
- Fredriksson, et al., “**Multiplex amplification** of all coding sequences within 10 cancer genes by Gene-Collector”, Nucleic Acids Research, 2007, vol. 35, No. 7 e47, 1-6.

'035 patent at 11:

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- Pinard, et al., “Assessment of **Whole Genome Amplification** induced Bias Through High-throughput, Massively Parallel Whole Genome Sequencing”, BMC Genomics, vol. 7:216, Aug. 23, 2006, 1-21.

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361. Likewise, Varley states:

This is the first method that couples multiplex PCR with sample-specific DNA barcodes and next-generation sequencing to enable highly multiplex mutation discovery in candidate genes for multiple samples in parallel.

Id. at Abstract.

Sample-specific DNA barcodes are then incorporated into the primers used for the final universal PCR by tailing the 5’ end with sample-specific DNA sequences and 454 sequencing primers.

Id. at 1845.

362. Further, Natera’s expert, Dr. Michael Metzker, has repeatedly acknowledged that amplification, including PCR, was well known in the art by 2011:

It was further well-known by 2008 that one could isolate, amplify and sequence cell-free genomic DNA from maternal blood to detect aneuploidy, including fetal trisomy 21....As such, one of ordinary skill would have had a reasonable expectation of success in using cell-free DNA from maternal blood in a multi-step PCR method (such as Fluidigm’s).

Ex. 12 ¶ 116 (emphasis added).

To perform NGS, sequencing libraries were often created from samples using PCR amplification. PCR-based library preparation was well-known and widely-used by the time the first application leading to the ’831 Patent was filed.

Id. ¶ 54.

As described above in this declaration, indexed sequences were routinely added by PCR as well. For example, Illumina described adding index sequences to its adaptor-ligated DNA fragments using a PCR enrichment step. Following additional library steps, each HapMap sample was pooled into a single tube and subjected to a PCR enrichment step using Illumina compatible primers.

Id. ¶ 67. Likewise, at deposition, Dr. Metzker admitted that the 2010 Fluidigm reference discloses multiplex PCR of over 9,000 targets, that a “person of ordinary skill in the art would understand how PCR works,” “one of ordinary skill would have understood how the PCR cycle works,” and that “[I]igation would be -- or PCR -- the two most common ways of [tagging cfDNA].” Ex. 6 at 87:24-88:6, 93:1-2, 94:6-7, 99:4-5, 114:24-115:1. Dr. Metzker acknowledged at trial that PCR “make copies of your target,” in other words, PCR is an amplification technique. Ex. 36 at 902:19-21.

363. Additionally, Natera’s expert, Dr. Quackenbush, testified that amplification was routine and conventional as early as 1985, well before 2011:

Q. And how far back were persons of skill in the art using PCR to do selected amplification?

A. So Kary Mullis, I described in 1985 Kary Mullis was doing it in 1985. It was a rapidly adopted technology because it was so powerful.

Ex. 11 at 88:14-18.

364. Dr. Quackenbush, also opined that the claimed amplification technique had been “universally adopted” as a standard tool by 2000:

PCR-based tools for genotyping SNPs, including for genotyping up to hundreds of thousands of SNPs, were commercially available as far back as 1995. ***By 2000, PCR had been universally adopted as a standard tool*** in applications of molecular biology, and it was already successful in the simultaneous detection of thousands of SNPs...***PCR was a well-established, conventional and indispensable tool*** for genetic testing that was routinely used to target and amplify specific, pre- selected genes for further study.

Ex. 20 ¶¶ 82-83 (emphasis added).

365. Dr. Quackenbush has also stated that he had significant experience with PCR techniques in the 1990s:

Starting in 1992, I ran a large program at the Salk Institute using PCR to assay polymorphic genetic markers to produce a map of human chromosome 11, a project that required tens of thousands of multiplexed PCR reactions. Between 1994 and 1996, while at Stanford University, I was responsible for the design and conduct of hundreds of thousands of PCR reactions for mapping the entire human genome and as part of developing a strategy for sequencing regions of human chromosomes 4 and 21. After joining The Institute for Genomic Research in 1997, I was responsible for projects involving hundreds of thousands of PCR reactions for genome sequencing and microarray analysis. And after joining the faculty at the Dana-Farber Cancer Institute and Harvard School of Public Health, I led large-scale projects analyzing gene expression in cancer and establishing high-throughput genome sequencing that required PCR and other amplification reactions from diverse biological samples, including cell lines, human tissue samples, circulating tumor cells, and circulating cell-free DNA. In all of these projects, I used standard and routine PCR applications for genotyping.

Id. ¶ 87. Further, Dr. Quackenbush has opined that “well before 2013 several techniques had been developed to generate or isolate copies of DNA molecules of interest to facilitate sequencing.” Ex. 7 ¶ 66. Likewise, Dr. Quackenbush cites to a document he describes as stating that PCR is among the most widely used approaches to target enrichment. *Id.* at ¶ 68.

366. Nor is multiplexing more than 25 loci in a single volume an inventive concept. The ’035 patent acknowledges that it was known in the art that up to 100 loci could be multiplexed in a single volume. D.I. 1-2 at 85:14-16 (“Methods described in the prior art used to multiplex more than 50 or 100 [loci] in one reaction volume followed by sequencing....”), 48:25-29 (“Also note that the general belief in the art is that multiplexing PCR for sequencing is limited to about 100 assays in the same well. Fluidigm and Rain Dance offer platforms to perform 48 or 1000s of PCR assays in parallel reactions for one sample.”). Likewise, the ’035 patent states that it was built on prior art, which included “fundamental methods” that “underlie the methods disclosed.” *Id.* at 93:41-58 (citing D.I. 52-5, D.I. 52-6, and D.I. 53-6). Varley provides an example of these

“fundamental methods,” and teaches multiplexing of 90 loci in a single volume. D.I. 52-5 at 1845 (“we designed oligonucleotides for **94 exons** from six genes that cause cancer when mutated in the germline (TP53, APC, MLH1, RB1, BRCA1, VHL)”). The ’035 patent mischaracterizes Varley as only teaching multiplexing of 9 loci in a single volume. D.I. 1-2 at 53-58 (“A method comprising multiplexing of an average of 9 assays for sequencing is described in” Varley.). As the ’035 patent acknowledges that 90- and 100-plexes were in the prior art, the use of 25-plexes cannot be an inventive concept.

367. Natera has twice taken the position that selective multiplex amplification was a well-understood, routine, and conventional method by 2009. Ex. 20 ¶ 8; Ex. D.I. 52-9 at 50 (“There is a good reason for this: the patentee admitted selective amplification was routine and conventional—so much so that it was unnecessary to provide any guidance on how to apply it to 1,000 SNPs.”) (citations omitted). Additionally, in *Illumina, Inc. v. Natera, Inc.*, Natera argued that methods of DNA amplification were known in the art by 2010. Ex. 34 at 5. If such techniques were conventional in 2009 and 2010, they were surely conventional by 2011.

368. In sum, the ’035 patent, Natera’s expert witnesses, and Natera’s own admissions show that the technique of amplifying cfDNA as claimed in the ’454 patent was well-known, routine, and conventional in 2011.

c. The ’035 Patent, Natera’s Expert Witnesses, and Natera’s Own Admissions State That DNA Sequencing Was a Well-Understood, Routine, and Conventional Method in 2011

369. Claim 1 of the ’035 patent recites “sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products, wherein the plurality of SNP loci comprises 25-2,000 loci associated with cancer.” D.I. 1-2 at claim 1. Gene sequencing was well known to a POSA.

370. Nowhere does the '035 patent state that the methods for sequencing described in the patent specification involve anything new, innovative, or novel. To the contrary, the '035 patent cites to a wealth of prior art publications describing routine, standard, sequencing techniques known in the art (all emphases below are added):

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- Binladen, J. et al., "The Use of Coded PCR Primers Enables **High-Throughput Sequencing** of Multiple Homolog Amplification Products by 454 Parallel Sequencing", PLOS One, Issue 2, Feb. 2007, 9 pages.
- Gnirke, A. et al., "Solution hybrid selection with ultra-long oligonucleotides for **massively parallel targeted sequencing**", Nature Biotechnology, vol. 27, No. 2, Feb. 2009, 182-189.
- Tewhey, R. et al., "Microdroplet-based PCR enrichment for **largescale targeted sequencing**", Nature Biotechnology, vol. 27, No. 11, Nov. 2009, 1025-1031.

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- Bentley, David R et al., "Accurate **Whole Human Genome Sequencing** Using Reversible Terminator Chemistry", Nature, 456, 6, 2008, 53-59.
- Bermudez, M. et al., "**Single-cell sequencing and mini-sequencing** for preimplantation genetic diagnosis", Prenatal Diagnosis, 23, 2003, 669-677.
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- Hollas, B. et al., “A stochastic approach to count RNA molecules using DNA **sequencing** methods”, Lecture Notes in Computer Science, vol. 2812, 2003, 55-62.

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- Leary, R. J. et al., “Development of Personalized Tumor Biomarkers Using **Massively Parallel Sequencing**”, Science Translational Medicine, vol. 2, No. 20, Feb. 24, 2010, 1-8.
- Li, R. et al., “SNP detection for **massively parallel whole-genome resequencing**”, Genome Research, vol. 19, 2009, 1124-1132.

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- Lo, Y.M. Dennis et al., “Maternal Plasma DNA **Sequencing** Reveals the Genome-Wide Genetic and Mutational Profile of the Fetus”, Science Translational Medicine, 2 (61), 2010, 13.
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- Ten Bosch, J., “Keeping Up With the **Next Generation Massively Parallel Sequencing** in Clinical Diagnostics”, Journal of Molecular Diagnostics, vol. 10, No. 6, 2008, 484-492.

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- You, Frank M. et al., “BatchPrimer3: A high throughput web application for PCR and **Sequencing** Primer Design”, BMC Bioinformatics, Biomed Central, London, GB, vol. 9, No. 1, May 29, 2008 (May 29, 2008), p. 253.

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- “Illumina, “Preparing Samples for **Sequencing** Genomic DNA”, Part # 11251892 Rev. A, 2007, 18 pages.
- “Illumina, “Technology: Solexa **Sequencing** Technology”, [https://web.archive.org/web/20070521_081517_/http://www.Illumina.com/pages.ilmn?I D203](https://web.archive.org/web/20070521_081517_/http://www.Illumina.com/pages.ilmn?I%203), May 21, 2007, 1 page.
- Robertson, G., et al., “Genome-wide profiles of STAT! DNA association using chromatin immunoprecipitation and **massively parallel sequencing**”, Nature Methods, Aug. 2007, 651-657.

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- Campbell, P. J., et al., “Subclonal phylogenetic structures in cancer revealed by **ultra-deep sequencing**”, PNAS, Sep. 2, 2008, 13081-13086.

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- Meyer, M, et al., “Illumina **Sequencing** Library Preparation for Highly Multiplexed Target Capture and **Sequencing**”, Cold Spring Harbor Protocols, vol. 2010, Issue 6, Jun. 2010, 1-10.

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- Li, et al., “Multiplex Padlock Targeted **Sequencing** Reveals Human Hypermutable CpG Variations”, Genome Research, vol. 19, No. 9, Jun. 12, 2009, 1606-1615.
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- Neve, B. et al., “Rapid SNP Allele Frequency Determination in Genomic DNA Pools by **Pyrosequencing**”, Bio Techniques, vol. 32, No. 5, May 1, 2002, 1138-1142.

’035 patent at 28:

- Ng, et al., “**Multiplex Sequencing** of Paired-end Ditags (MS-PET): A Strategy for the Ultra-high-throughput Analysis of Transcriptomes and Genomes”, Nucleic Acids Research, vol. 34, No. 12, Jul. 13, 2006, 1-10.
- Pinard, et al., “Assessment of Whole Genome Amplification-induced Bias Through **High-throughput, Massively Parallel Whole Genome Sequencing**”, BMC Genomics, vol. 7:216, Aug. 23, 2006, 1-21.
- Pourmand, et al., “**Multiplex Pyrosequencing**”, Nucleic Acid Research, vol. 30, No. 7, Apr. 1, 2002, 1-5.
- Smith, et al., “Rapid Whole-genome Mutational Profiling using **Next-generation Sequencing** Technologies”, Genome Research, vol. 18, Sep. 4, 2008, 1638-1642.
- Stiller, et al., “Direct Multiplex Sequencing (DMPS)-A Novel Method for Targeted **High-throughput Sequencing** of Ancient and Highly Degraded DNA”, Genome Research, vol. 19, No. 10, Jul. 27, 2009, 1843-1848.
- Voelkerding, et al., “**Next-generation Sequencing**: From Basic Research to Diagnostics”, Clinical Chemistry, vol. 55, No. 4, Apr. 1, 2009, 641-658.

- Wasson, Jon et al., “Assessing Allele Frequencies of Single Nucleotide Polymorphisms in DNA Pools by **Pyrosequencing** Technology”, BioTechniques, vol. 32, No. 5, May 1, 2002, 1144-1152.
- Wiedmann, Ralph T. et al., “SNP Discovery in Swine by Reduced Representation and **High Throughput Pyrosequencing**”, BMC Genetics, vol. 9, Article No. 81, Dec. 4, 2008, 1-7.

’035 patent at 29:

- Xie, et al., “CNV-SEQ, A New Method to Detect Copy Number Variation Using **High-throughput Sequencing**”, BMC Bioinformatics, vol. 10:80, Mar. 6, 2009, 1-9.
- Zhou, et al., “**Pyrosequencing**, A High-throughput Method for Detecting Single Nucleotide Polymorphisms in the Dihydrofolate Reductase and Dihydropteroate Synthetase Genes of Plasmodium Falciparum”, Journal of Clinical Microbiology, vol. 44, No. 11, Nov. 1, 2006, 3900-3910.

371. Further, Varley states:

This is the first method that couples multiplex PCR with sample-specific DNA barcodes and next-generation sequencing to enable highly multiplex mutation discovery in candidate genes for multiple samples in parallel.

D.I. 52-5 at Abstract.

Sample-specific DNA barcodes are then incorporated into the primers used for the final universal PCR by tailing the 5’ end with sample-specific DNA sequences and 454 sequencing primers.

Id. at 1845.

372. Likewise, Natera’s expert, Dr. Michael Metzker, has repeatedly acknowledged that sequencing was well known in the art by 2011:

As discussed above, in the relevant timeframe, next-generation sequencing was in widespread use....Further, a kit for making a sequencing library for use with Solexa’s sequencer was commercially available in 2008, underscoring the routine nature of sequencing library preparation. Thus, one of ordinary skill would have expected success in being able to sequence cell-free DNA found in maternal blood.

Ex. 12 ¶ 115.

It was further well-known by 2008 that one could isolate, amplify and sequence cell-free genomic DNA from maternal blood to detect aneuploidy, including fetal trisomy 21....As such, one of ordinary skill would have had a reasonable

expectation of success in using cell-free DNA from maternal blood in a multi-step PCR method (such as Fluidigm's). One of ordinary skill would have expected reasonable success in identifying sequences from particular chromosomes, because the human genome had already been sequenced by the relevant timeframe. Cell-free genomic DNA from maternal blood had also previously been successfully amplified and sequenced to detect aneuploidy, including fetal trisomy 21, trisomy 18 and trisomy 13. This typically involved amplifying DNA from a suspected chromosome and a reference chromosome and detecting whether levels of sequences of the suspect chromosome were elevated. Given the success in using cell-free DNA to perform amplification and sequencing, one of ordinary skill would have had a reasonable expectation of success to employ cell-free DNA from maternal blood in the Fluidigm method with the goal of testing for chromosomal aneuploidy.

Ex. 12 ¶ 116 (citations omitted).

Since their introduction, NGS platforms have allowed for high-throughput sequencing of nucleic acids on a genome-wide scale and highly multiplexed gene-specific sequencing.

Ex. 12 ¶ 41.

The first NGS platform to achieve widespread commercial use was 454 Life Sciences Corporation's sequencer, also known as Genome Sequencer 20 ("GS20"). The GS20 sequencer first became commercially available in 2005 and produced average read-lengths of ~100 bases.

Ex. 12 ¶ 42.

The Genome Analyzer II ("GAII") was developed by Solexa and released in late 2006. After the acquisition of Solexa by Illumina in 2007, this massively parallel sequencing platform achieved widespread commercial use.

Ex. 12 ¶ 45.

Not only was the concept of creating an indexed DNA sequencing library routine and commonplace, the concept of using cell-free DNA from maternal blood to detect chromosomal aneuploidy, such as trisomy 21, the underlying cause of Down syndrome was equally well-known in the art.

Ex. 12 ¶ 56.

As of January 2010, Illumina's GA sequencer was the most commonly used next-generation sequencer....The GA instrument was capable of sequencing a sample from an individual or multiple samples from many different individuals.

Ex. 12 ¶ 62.

As described above in this declaration, indexed sequences were routinely added by PCR as well. For example, Illumina described adding index sequences to its adaptor-ligated DNA fragments using a PCR enrichment step. Following additional library steps, each HapMap sample was pooled into a single tube and subjected to a PCR enrichment step using Illumina compatible primers.

Ex. 12 ¶ 67.

373. At trial, Dr. Metzker admitted that he “first started working with PCR and DNA sequencing technologies in 1987,” that he published on NGS as early as 1994, that NGS technologies became commercially available in 2005, that between 2005 and 2010 there were “a lot of breakthroughs in new technologies” in NGS, and that by 2011 there were “numerous [MGS] instruments on the market.” Ex. 36 at 974:14-16, 888:21-889:1, 894:12-14, 900:22-901:2, 957:2-6. Likewise, Dr. Metzker wrote a 2010 review article that discussed, *inter alia*, that people had “begun sequencing complete human genomes.” *Id.* at 900:22-901:22; Ex. 37 at 6.

374. Furthermore, Dr. Quackenbush posits that high-throughput sequencing was routine and conventional as of 2010. In his declaration, Dr. Quackenbush states that the sequencing method, claimed in the '035 patent was well-known, commercially available, and routinely used by researchers well before 2011:

[A] method called pyrosequencing, which performs ***sequencing-by-synthesis***, was developed and first published in 1993. It was the foundation for different companies. PYROSEQUENCING AB, out of Uppsala Sweden, launched its first commercial automated pyrosequencing instrument in 1999....Based on the machines made available by Pyrosequencing, by the year 2000, pyrosequencing had established itself as a ***standard and conventional*** means for multiplex or ***high-throughput sequencing***, including for genotyping SNPs.

Ex. 20 ¶103 (citing Marsh 2007, Ahmadian 2000; Nordstrom 2000; Ronaghi 2001).

Another ***high throughput sequencing-by-synthesis*** method developed by a group out of Cambridge University in 1998 became the foundation for a company called Solexa (later acquired by Illumina, Inc.)....As the group that developed this

technology reported in Margulies 2005, the instrument branded as the Genome Analyzer could “sequence 25 million bases, at 99% or better accuracy, in one four-hour run,” B11305- 1309 (Margulies 2005) at B1305 (Abstract), and had an average read length of 108 bases

Id. ¶ 104 (citing Marguiles 2005).

As another example, a high throughput sequencing method co- developed by the named inventor Dr. Quake became the foundation for his company, Helicos, which was founded in 2003, and on which I was a member of the Scientific Advisory Board.

Id. ¶ 106.

375. I understand that Dr. Quackenbush opined that in 2005, there was the expectation that high-throughput sequencing would generate large quantities of data to understand cancer—an expectation that was quickly realized:

For example, when I began working in the biological sciences at the Salk Institute in 1992, the first molecular biology techniques that I learned were PCR and Sanger DNA sequencing. As part of my move to Stanford University in 1994, I was tasked with developing a new large-scale DNA sequencing method based on PCR mapping of transposon insertions. My recruitment to the Dana-Farber Cancer Institute in 2005 was largely based on the expectation that high throughput DNA sequencing would soon generate unprecedented quantities of data that could be used to understand cancer. And that expectation very quickly was realized throughout the genomics field.

Id. ¶ 87.

376. Dr. Quackenbush also noted that in 2005 there was a rapid explosion in the use of sequencing by synthesis instruments:

Due to the rapid explosion in use of sequencing-by-synthesis instruments including the 454 sequencer in 2005, the Illumina Genome Analyzer and Applied Biosystems SOLiD sequencer in 2006, and others shortly thereafter, my group and I quickly became involved in multiplex / high-throughput (or Next Generation Sequencing (“NGS”)) DNA sequencing.

Id. ¶ 95.

377. According to Dr. Quackenbush, there were a number of commercially available sequencers. *See, e.g., Id.* at ¶¶ 103-113 (describing, various commercially available sequencing platforms, including, pyrosequencing, technology available by Helicos Biosciences Corporation, technology available by 454 Lifesciences, Clonal Single Molecule Array (Solexa, Inc.) or sequencing-by-synthesis (SBS) utilizing reversible terminator chemistry; AnyDot chips (Gonovox, Germany); sequencers such as Illumina Genome Analyzer, etc.). Further, Dr. Quackenbush has opined that by “2010, whole-genome sequencing to 30x coverage was routine on the commercially available HiSeq 2000.” Ex. 7 ¶ 64. He likewise opined that by “approximately 2008, techniques for NGS were well-known and commercially available,” that “using NGS, the entire human genome can now be sequenced on a single instrument in a single day,” and that the “development of NGS made more widely available ‘[t]he ability to sequence entire human genomes.’” *Id.* ¶¶ 60-61, 63 (quoting Lam et al. *Performance comparison of whole-genome sequencing platforms*, *Nature Biotechnology* 30:78 (2012)).

378. Natera, through its expert’s declaration, has taken the position that sequencing to identify SNVs was a well-understood, routine, and conventional method by 2009. Ex. 20 ¶ 93 (describing that determining SNPs (SNVs) by “whole genome sequencing or exome sequencing” in 2009 was routine and conventional by reference to patents pre-dating 2009). Additionally, in *Illumina, Inc. v. Natera, Inc.*, Natera argued that methods of sequencing were known in the art by 2010. Ex. 34 at 5 (“the ’831 patent does not recite any new or unknown type of sequencing library”). If such techniques were conventional in 2009 and 2010, they were surely conventional by 2011.

379. I understand that Natera has taken the position that references cited in the prosecution histories of the ’454 patent establish that a POSA would have been known of ways to

perform sequencing before 2005. *See* Ex. 29 at 12 (citing Exs. 31-33). Further illustrating that the claimed method was routine, Natera argued that the “patentees knew about different types of sequencing-by-synthesis...when they drafted the claims, evidenced by cites to Jarvie and other references during prosecution.” *See* Ex. 29 at 16 (citation omitted).

380. In sum, the '035 patent, Natera's expert witnesses, and Natera's own admissions show the method of high-throughput sequencing as claimed in the '035 patent was well-known, routine, and conventional in 2011.

d. Claims 12 and 13 Add Only Conventional Steps

381. It is my opinion that claims 12 and 13 fail to add an inventive concept and add only conventional steps. Claim 12 depends from claim 1 and simply specifies that the “one or more universal tail adaptors” comprise “a first universal tail adaptor and a second universal tail adaptor.” D.I. 1-2 at claim 12. Indeed, “universal tail adaptors” were so well known in the art that the '035 patent specification did not even include a single reference to them. Ex. 6 at 34:15-35:1. Claim 13 depends from claim 12 and adds that the first and second universal tail adaptors are comprised of first and second primers. Primers were likewise ubiquitous in the art. *See, e.g.*, D.I. 1-2 at 7-26 (citing to a wealth of prior art publications describing the use of primers); D.I. 52-5 at 1845 (Sample-specific DNA barcodes are then incorporated into the *primers* used for the final universal PCR by tailing the 5' end with sample-specific DNA sequences and 454 sequencing *primers*.) (emphasis added); Ex. 12 ¶ 67 (discussing the use of “Illumina compatible primers”). Neither claim 12 nor claim 13 add an inventive concept.

382. For the reasons discussed above, it is my opinion that the Asserted Claims of the '035 patent are method of detection claims, that these only detect cfDNA in a biological sample,

which is a natural phenomenon, and that (based on the '454 patent, Natera's expert witnesses, and Natera's own admissions) the detecting is accomplished with no meaningful non-routine steps. There is nothing about the claimed steps, individually or in combination, that is unconventional.

XII. INVALIDITY OF THE '454 PATENT FOR IMPROPER INVENTORSHIP

383. As I explain above, the claims of the '454 patent are not inventive, but are rather invalid as obvious in view of multiple prior art references. To the extent the claims of the '454 patent are deemed to reflect a bona fide invention, however, it is my opinion that they are invalid for failure to name the proper inventors. Specifically, as I explain below, the claimed approach based on whole exome sequencing was invented not by Natera alone, but rather in collaboration with researchers at University College London ("UCL"), including, for instance, Dr. Miriam Jamal-Hanjani.

A. Disclosure Relating to Whole Exome and Whole Genome Sequencing In The '454 Patent

384. The independent claims of the '454 patent recite the step of:

performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

D.I. 1-1 at claims 1, 14. Thus, thus the claims recite the use of either "whole exome" or "whole genome" sequencing.

385. There is no description of "whole genome sequencing" in the '454 patent, a point that I discuss further below in connection with written description. Although the description for Figure 51B refers to "whole genome sequencing," this is an error because Figure 51 is actually only "whole exome" sequencing. As to "whole exome" sequencing, it is my opinion that the '454 patent has limited written description. Specifically, it is my opinion that the sole support for "whole exome" sequencing appears in the '454 patent's Example 13.

386. Specifically, Example 13 provides as follows:

Two to three biopsies from various regions from the entire cancerous lung were taken from each patient (FIG. 51A). Each biopsied sample was assayed by whole exome sequencing (Illumina HiSeq200; Illumina, San Diego, Calif.), followed by Amp-15 liSeq® sequencing (Ion Torrent, South San Francisco, Calif.) on a PGM® for identification of underlying clonal heterogeneity. Following sequencing and SNV analyses, the variant allele frequency (VAF) was determined for each biopsy sample (FIG. 51B).

Id. at 168:10-19. I have reviewed the entirety of the '454 patent in the context of identifying written description support in the disclosure for whole exome sequencing and identified nothing other than the foregoing in Example 13.

B. The '454 Patent Example 13 and the Jamal-Hanjani Thesis

387. I have reviewed and compared disclosures of the '454 patent (D.I. 1-1) and the Jamal-Hanjani Thesis (Ex. 38).

388. It is my opinion that the limited written description for “whole exome” sequencing in the '454 patent is the same as a disclosure of research based on the use of “whole exome” sequencing that is appears in the Jamal-Hanjani Thesis.

389. Based on comparison of the disclosures, I focus my discussion below on “Section 5.4 Multiplex PCR and targeted HiSeq sequencing” of the Jamal-Hanjani Thesis. Ex. 38 at 155-160.

390. Section 5.4 includes Table 22 (Clinical characteristics of the patient cohort), Table 23 (List of selected mutations for the Natera approach), and Figure 49 (Concordance between VAFs). Ex. 38, 155 (Table 22), 156 (Figure 49), 157-158 (Table 23).

Patient ID	Age	Gender	Histology	Stage	Vascular invasion	Pleural invasion	Smoking status (pack years)
L012	69	F	LUSC	IB	Y	Y	Smoker (40)
L013	68	F	LUSC	IB	Y	Y	Smoker (50)
L015	68	M	LUSC	IA	N	N	Smoker (100)
L017	61	F	LUAD	IIB	Y	N	Smoker (48)

Table 22 Clinical characteristics of the patient cohort

Abbreviations: LUAD, lung adenocarcinoma; LUSC, lung squamous cell carcinoma.

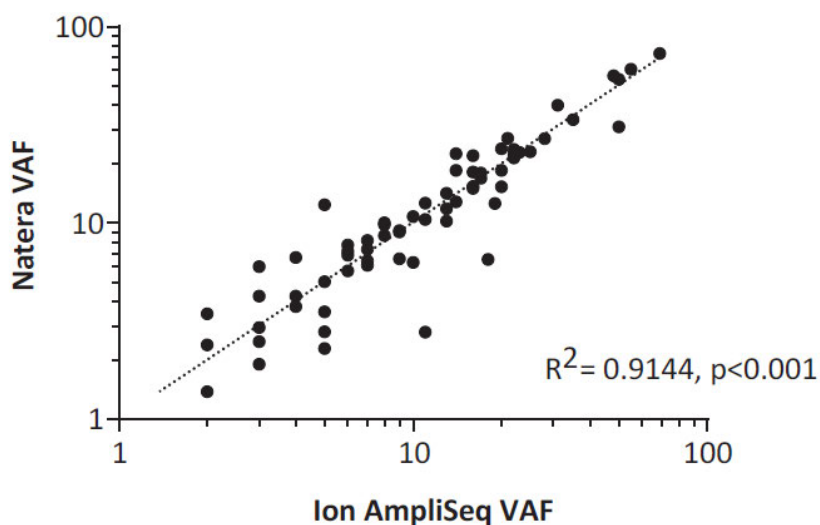


Figure 49 Concordance between VAFs

Concordance between individual mutation variant allele frequencies (VAFs) and mean VAF (vertical line) identified by prior sequencing (WES and Ion AmpliSeq) and the Natera approach by direct comparison (A), and by linear regression modeling (B).

Patient	Gene (amino acid change)	WES/Ion (VAF %)			AmpliSeq			Natera (VAF %)			cfDNA (VAF %)	Truncal/ Branch
		R1	R2	R3	R1	R2	R3	R1	R2	R3		
L012 LUSC Stage IB Smoker (40)	BRIP1 (R173C)	14.0	6.0	8.0				22.6	7.7	10.1	3.71	Truncal
	CARS (T51A)	22.0	11.0	16.0				22.4	10.5	18.2	5.02	Truncal
	CIC (E61X)	0.1	nd	7.0				nd	nd	8.2	1.71	Branch
	FAT1 (D924Y)	8.0	4.0	1.2				9.8	4.3	0.8	0.57	Branch
	KDM6A (R4079K)	10	5.0	0.8				6.3	3.5	nd	0.28	Branch
	MLLT4 (E478Q)	9.0	3.0	0.3				9.0	4.3	0.7	0.95	Branch
	NFE2L2 (E1142D)	69.0	31.0	50				73.1	39.9	53.9	23.25	Truncal
	RASA1 (E183X)	6.5	nd	0.7				7.4	nd	0.4	nd	Branch
	TP53 (R136H)	23.0	9.0	17.0				22.9	9.2	17.0	4.89	Truncal
	TP53 (Y181C)	22.0	8.0	16.0				21.5	8.7	22.1	5.77	Truncal
L013 LUSC Stage IB Smoker (50)	EGFR (G719A)	21.4	24.8	55.2				27.1	23.1	60.8	1.16	Truncal
	EGFR (D761Y)	20.1	17.3	48.0				23.9	18.0	56.3	1.09	Truncal
	HERC4 (N217K)	0.1	3.0	6.0				0.2	1.9	5.7	nd	Branch
	JAK2 (K33X)	0.2	11.1	6.8				0.3	2.8	6.1	nd	Branch
	MLL3 (K589R)	0.2	0.2	4.1				nd	nd	6.7	nd	Branch
	MSH2 (Q444H)	4.8	4.2	11.0				5.0	3.3	12.7	nd	Truncal
	MTOR (Q838E)	2.0	0.6	3.0				2.4	1.4	6.0	nd	Truncal
	PLCG2 (E525X)	5.1	1.7	10.5				2.8	1.7	6.9	nd	Truncal
	TP53 (P60S)	7.0	4.3	15.6				6.1	3.8	15.4	0.4	Truncal
L015 LUSC Stage IA Smoker (100)	ALK (P234R)	6.0	2.0	NS				7.2	1.4	NS	nd	Truncal
	GABRG1 (I279F)	13.0	0.8	NS				11.8	2.0	NS	nd	Truncal
	KDM6A (S539C)	18.0	6.0	NS				6.5	0.9	NS	0.17	Truncal
	MLL2 (E1186X)	5.0	0.3	NS				12.4	nd	NS	nd	Branch
	ROS1 (Y891C)	10.0	3.0	NS				10.8	2.9	NS	0.15	Truncal
	SLC39A4 (A546T)	14.0	nd	NS				18.6	nd	NS	nd	Branch
	TP53 (G199X)	19.0	3.0	NS				12.6	2.5	NS	nd	Truncal

Ex. 38 at 155 (Table 22), 156 (Figure 49), 157-158 (Table 23).

391. Jamal-Hanjani Thesis Table 22 sets forth data mirroring that in '454 patent Figure 51A. Ex. 38 at 155 (Table 22); D.I. 1-1 at Fig.51A.

392. Jamal-Hanjani Thesis Table 23 sets forth data regarding WES/Ion Ampliseq (VAF %) and Natera (VAF%) mirroring that in '454 patent Figures 51B and 53A. Ex. 38 at 157-158 (Table 23); D.I. 1-1 at Figs 51B, 53A-B.

393. Jamal-Hanjani Thesis Figure 49 appears the same as '454 patent Figure 54B.

394. I have also considered other relevant portions of the Jamal-Hanjani Thesis, including lists of genes location referred to in Example 13, which are identical. Ex. 38 at 68-70; D.I. 1-1 at Figs.51B, 53A-B.

395. Based on my comparison of the work reported in the Jamal-Hanjani Thesis and '454 patent Example 13, it is my opinion that research reported in the Jamal-Hanjani Thesis is disclosed in the '454 patent in Example 13.

C. The UCL Contribution To The Claims

396. As I explain above, the sole support in the specification for “whole exome” sequencing as recited in the claims appears in Example 13, and this example in the patent matches the disclosure in Chapter 5 of the Jamal-Hanjani thesis. For several reasons, it is evident that researchers at UCL, including at least Dr. Jamal-Hanjani, contributed to the conception of the approach in Example 13, including the use of whole exome sequencing and analysis of cell-free DNA to detect tumor-specific mutations.

1. The Jamal-Hanjani Thesis Itself

397. I start with the Jamal-Hanjani thesis itself. The fact the content of Example 13 matches Chapter 5 of the Jamal-Hanjani Thesis is evidence that she made a significant contribution to the claimed inventions of the '454 patent.

398. Beyond this, the thesis states that Dr. Hanjani wrote the protocol for the TRACERx study (without input from Natera), which started in April 2014:

I wrote and developed the protocol for this study under the guidance of my supervisor during the production of this thesis, with the input of the TRACERx consortium and the support of the UCL Cancer Trials Centre (Alan Hackshaw, Yentig Ngai, and Natasha Iles). This study started recruitment in April 2014, and is currently open in London, Manchester, Leicester, Birmingham, Aberdeen, and Cardiff.

Ex. 38 at 26. The protocol included determining whether cfDNA includes mutations found in tumor sequencing. *Id.* at 25 (“Surgically resected primary NSCLC tumours and associated lymph nodes, surplus to diagnostic requirements, will be subjected to multi-region sampling and subsequent WES and/or WGS.”). The protocol also involved tracking those mutations over time in cell free DNA to monitor residual disease. *Id.* at 26 (“To determine if cfDNA and CTCs can be used to track actionable mutations to guide therapeutic intervention, monitor residual disease and predict tumour recurrence.”).

399. Given that Dr. Hanjani wrote the protocol and research plan involving whole exome sequencing and monitoring of cell-free DNA in mutations (without input from Natera), it seems clear that she would have made a significant contribution to the alleged inventions of the '454 patent, which include steps pertaining to these very concepts.

400. Consistent with the foregoing, the Materials and Methods section of the Jamal-Hanjani thesis describes how Dr. Hanjani provided variants and samples for to collaborators, including Natera, for processing and that it was her that lab analyzed the results:

2.12 cfDNA analyses

2.12.1 Selection of mutations

Non-silent mutations identified by WES and subsequently validated by Ion AmpliSeq sequencing were considered for detection in cfDNA. The majority of the mutations investigated were SNVs, but in some cases indels were tested and in one case an EML4-ALK translocation was tested (collaboration with Illumina). All approaches involved multiplex PCR and PCR primers were designed using the specified genomic coordinates for the selected mutations. In order to address the question as to whether the heterogeneous genetic landscape of a tumour could be explored using cfDNA, both truncal and branch mutations were selected. cfDNA analyses involved collaborations with companies outside of our laboratory (as described below), whereby cfDNA extracted at diagnosis from 2ml of plasma for each patient was sent to these companies along with specified mutations for detection. *The results of these experiments were analysed in our laboratory.* Significant associations between VAFs for selected mutations in these analyses were tested for using the Mann-Whitney U test.

Id. at 53. The foregoing further shows that Dr. Jamal-Hanjani made a significant contribution to the claims with regard to sequencing of tumor mutations and subsequent monitoring of such mutations in cell-free DNA. Indeed, it appears she was not just conceiving of the experiments, but also participating in the analysis of the results to confirm the utility of the approach.

2. Testimony From Dr. Zimmermann

401. I also understand that Natera made Dr. Bernhard Zimmerman, a named inventor on Natera's patents, available to testify as its corporate representative regarding Natera's collaboration with UCL. His testimony further confirms my opinions.

402. For instance, section 5.4 of the Jamal-Hanjani thesis discloses work to detect mutations in cfDNA. As the Jamal-Hanjani thesis explains, the mutations used were those that were identified using whole exome sequencing. *See* Ex. 38 at 158 (Table 23 caption, "List of mutations and variant allele frequencies (VAFs) identified using WES/AmpliSeq sequencing and the Natera approach."). As Dr. Zimmerman explains, this work was not Natera's alone, but was the product of collaboration between Natera and Dr. Jamal-Hanjani:

Q. Okay. And 5.4 this is the collaboration between Natera and Hanjani; is that correct?

A. And what.

Q. 5.4. Section 5.4 on page 155?

A. That is correct, yes.

Ex. 35 (Zimmermann Tr.) at 55:10-14.

403. As Dr. Zimmerman stated, the whole exome sequencing in Example 13 was done by Jamal-Hanjani and Example 13 was a product of collaboration between Natera and UCL, wherein Natera allegedly did the cell-free DNA analysis:

Q. The whole exome sequencing to identify the signatures, that was what? Was performed by Dr. Jamal-Hanjani and her team, correct? I think you were pretty clear about that before.

A. Yes.

Q. Okay. And in terms of working on Example 13 you'll agree that was the product of collaborative work between UCL and Natera?

A. Well, it was a product of a collaboration, right where we did -- did -- we performed the analysis of the cell-free DNA.

Id. at 87:13-20. He confirmed this multiple times:

Q. Do you see the research plan that was exchanged between Dr. Jamal-Hanjani and Robert Pelham has the reference to "whole exome sequencing" in the third paragraph about midway through it? Do you see it right before Footnote 3?

A. Yeah.

Q. And then the attribution for the whole exome sequencing, that's -- in this plan is to the Jamal-Hanjani PLOS publication?

A. Yes.

Id. at 94:1-10.

In view of the foregoing evidence, it is my opinion that researchers at UCL would have made a significant contribution to the claims of the '454 patent, including by conceiving

of the use of whole-exome sequencing of tumor tissue followed by the analysis of cell-free DNA to detect tumor-specific mutations. Also, I am informed that conception, as demonstrated here, indicates inventorship.

XIII. INVALIDITY UNDER 35 U.S.C. § 112—LACK OF WRITTEN DESCRIPTION

404. It is my opinion that to the extent the claims of the '454 and '035 patents are not invalid as being obvious, they are invalid for lack of written description.

405. As to the '454 patent specifically, I note that independent claims of the '454 patent recite the step of:

performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

D.I. 1-1 at claims 1, 14. Thus, thus the claims recite the use of either “whole exome” or “whole genome” sequencing.

406. There is, however, no description of “whole genome sequencing” in the '454 patent. Although the description for Figure 51B refers to “whole genome sequencing,” this is an error because Figure 51 is actually only “whole exome” sequencing. Dr. Zimmerman confirmed that this is the case:

[REDACTED]

[REDACTED]

[REDACTED]

407. One can further confirm this by comparing the data that appears in Fig. 51b of the '454 patent to the data that appears in Table 23 of the Jamal-Hanjani thesis. As one can see, the

data in the Jamal-Hanjani Thesis was generated by “WES,” which is “whole exome sequencing,” not “whole genome sequencing.” Ex. 38 at 157 (Table 23). This data matches exactly what is in Figure 51b of the patent, confirming that Figure 51b of the patent is showing whole exome sequencing data, not whole genome sequencing data.

408. Thus, the claims lack written description because there is no description in the '454 patent of the “whole genome sequencing” claim element.

409. The claims of the '035 patent and '454 patents are also invalid for failure to describe techniques of PCR amplification without the use of techniques for selecting primers.

410. The claims of the '035 and '454 patents both recite steps related to performing PCR on multiple nucleic acid targets in the same reaction volume at the same time, a process referred to as “multiplex PCR.” *See* D.I. 1-2 at 2:65-3:4; D.I. 1-1 at 58:40-46 (“multiplex amplification reaction...such as PCR”).

411. For the '454 patent, claim 1 recites

performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume

D.I. 1-1 at claim 1.

412. For the '035 patent, claim 1 recites

amplifying the tagged products one or more times to generate final amplification products, wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume

D.I. 1-2 at claim 1.

413. It is my understanding that in a co-pending case involving organ transplant, Natera put forth the position that their patents, which are in the same family as the patents in this case,

cover techniques for multiplex PCR with an unlimited number of randomly selected targets (i.e., without the use of any techniques for loci/primer selection) that supposedly yields usable nucleic acid for high throughput sequencing. The claims for the patents in this case also include techniques for multiplex PCR. I understand that Natera may be bound to that position in this case. If so, the patents in this case, nor any of the family member patents to the patents in this case, disclose such a technique for multiplex PCR. Rather, the inventors were in possession solely of techniques that involved target loci selection and primer design for achieving multiplex PCR.

414. As an initial matter, as I explain above in connection with obviousness, the individual concepts recited in the claims, such as multiplex amplification, molecular barcodes, and sequencing amplicons were all known in the art. Natera has acknowledged that these were not new things, as I explain in Section X. Therefore, to the extent there is anything inventive in the claims of the asserted patents, it must reside in the combination of recited elements. Having reviewed the patents, however, I do not believe there is any embodiment that discloses the elements of the claimed inventions of the '454 or '035 Patents as arranged in the claims. This alone establishes that the inventors were not in possession of the claimed inventions.

415. For example, the '454 patent does not disclose an example that discusses sequencing a tumor sample and then obtaining any depth of read for sequenced cell-free DNA. As for the '035 patent, claim 1 requires the use of "universal tail adaptors," which is not a term used or defined anywhere in the specification, let alone in conjunction with the other steps of claim 1 in any kind of example. This establishes that the inventors were not in possession of the claimed inventions.

416. As discussed, to the extent the claims are not invalid as obvious, the specification also makes clear to the skilled artisan that the inventors were solely in possession of techniques that involved target loci selection and primer design.

417. First, as recited above, the claims of the '454 and '035 patents recite *inter alia* steps related to performing PCR on multiple nucleic acid targets in the same reaction volume at the same time, a process referred to as “multiplex PCR.” Specifically, claim 1 of the '454 patent, the sole asserted independent claim, recites “performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor specific SNV mutation from cell-free DNA.” D.I. 1-1 at 171:33-34. Likewise, claim 1 of the '035 patent, the sole asserted independent claim, recites amplifying tagged nucleic acid samples “wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume” and sequencing the plurality of SNP loci, wherein the plurality comprises “25-2,000 loci associated with cancer.” D.I. 1-2 at 249:43-62.

418. As an example of disclosures of only techniques that involved target loci selection and primer design, the very first sentences of the Detailed Description of the invention explained that the alleged invention is about targeting sequences for amplification to avoid undesirable side products like primer dimers. As the specification explains, the “present invention is based in part on the surprising discovery that often only a relatively small number of primers in a library of primers are responsible for a substantial amount of the amplified primer dimers that form during multiplex PCR reactions.” *Id.* at 46:37-41. Accordingly, the specification provides that “[m]ethods have been developed to select the most undesirable primers for removal from a library of candidate primers.” *Id.* at 46:41-43. “By reducing the amount of primer dimers to a negligible amount...these methods allow the resulting primer libraries to simultaneously amplify a large

number of target loci in a single multiplex PCR reaction.” *Id.* at 46:43-47. As such, the specification discloses that because “the primers hybridize to the target loci and amplify them rather than hybridizing to other primers and forming amplified primer dimers, the number of different target loci that can be amplified is increased.” *Id.* at 46:47-451.

419. Further confirmation that the inventors were solely in possession of techniques that involved target loci selection and primer design is established in the specification’s disclosure that it is essential to remove such primer-dimers by selecting primers to avoid primer dimers: “[a]t high multiplexing it is not possible to eliminate all spurious interactions, but *it is essential* to remove the primers or pairs of primers with the highest interaction scores in silico as they can dominate an entire reaction, greatly limiting amplification from intended targets.” *Id.* at 54:46-50; D.I. 1-1 at 108:18-22. Likewise, “when multiple pairs are added to the same PCR reaction, non-target amplification products may be generated, such as amplified primer dimers. The risk of generating such products increases as the number of primers increases. These non-target amplicons significantly limit the use of the amplified products for further analysis and/or assays. Thus, improved methods are needed to reduce the formation of non-target amplicons during multiplex PCR.” D.I. 1-2 at 3:6-14; *see also id.* at 47:53-55, 96:27-30; *see also* D.I. 1-1 at 106:2-6. Additionally, the patents explain that without target loci selection and primer design techniques, the PCR reaction yields essentially nothing but primer dimer: “Sequencing of a 1042-plex without design and selection of assays resulted in >99% of sequences being primer dimer products.” D.I. 1-2 at 96:28-30. If the inventors were in possession of techniques that did not require target loci selection and primer design for use with the claimed techniques, they would not state that such techniques are “essential” and point out that without the use of such techniques the yield is almost nothing but primer dimer.

420. In fact the specification relies on the avoidance of primer side products to distinguish the prior art. The specification identifies the problem of “[h]ighly multiplexed PCR [that] can often result in the production of...unproductive side reactions such as primer dimer formation.” *Id.* at 48:7-10. Positing the alleged invention as a solution to this problem, the specification provides that the invention is an “alternative to microarrays” for sequencing, since the invention provides for the “high level of multiplexing with minimal nontarget amplicons that has now been achieved.” *Id.* at 47:40-49.

421. Moreover, the specification frames the alleged invention’s removal of “problematic primers, that is, those primers that are particularly likely to f[o]rm dimers” as the element that “unexpectedly enabled extremely high PCR multiplexing[.]” *Id.* at 48:14-17; *see also id.* at 48:32-36 and D.I. 1-1 at 105:64-106:1 (“Empirical data indicate that a small number of ‘bad’ primers are responsible for a large amount of non-mapping primer dimer side reactions. Removing these ‘bad’ primers can increase the percent of sequence reads that map to targeted loci.”); D.I. 1-2 at 48:30-32 (disclosing that there “are a number of ways to choose primers for a library where the amount of non-mapping primer dimer...products are minimized.”). In this way, the patentee distinguished the invention from the prior art by directly connecting the removal of the primers that cause primer dimers as something “unexpected.” *Id.* at 48:10-13.

422. The increase in amplification accuracy and efficiency is the stated solution to the problem the patentee identified in conventional DNA amplification techniques in the Background. *Id.* at 2:64-3:13. For example, the specification discloses that, in “systems such as sequencing, where performance significantly degrades by primer dimers...greater than 10, greater than 50, and greater than 100 times higher multiplexing than other described multiplexing has been achieved”

due to the selection of primers that “hybridize to the target loci and amplify them rather than hybridizing to other primers and forming primer dimers[.]” *Id.* at 48:18-24.

423. The individual embodiments disclosed in the specification again emphasize avoiding primer side products:

In various embodiments, less than 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% ***of the amplified products are primer dimers.***

D.I. 1-2 at 8:60-63 (emphasis added).

In some embodiments, the library includes primers that simultaneously amplify at least 25; 50; 75; 100; 300; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 15,000; 19,000; 20,000; 25,000; 30,000; 40,000; 50,000; 75,000; or 100,000 ***different target loci such that less than 60, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% of the amplified products are primer dimers.***

Id. at 19:1-9 (emphasis added).

In some embodiments, ΔG values for each possible combination of two primers (***each possible primer dimer***) in a library are all equal to or greater than -20, -18, -16, -14, -12, -10, -9, -8, -7, -6, -5, -4, -3, -2, or -1 kcal/mol.

Id. at 19:54-62 (emphasis added).

In some embodiments, (i) less than 60% of the amplified products ***are primer dimers*** and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products ***are primer dimers*** and at least 60% of the amplified products are target amplicons, (iii) less than 20% of the amplified products ***are primer dimers*** and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products ***are primer dimers*** and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products ***are primer dimers*** and at least 95% of the amplified products are target amplicons.

Id. at 22:3-14 (emphasis added).

424. The examples in the patents further confirm that the inventors were in possession solely of techniques that utilized target loci selection and primer design for multiplex PCR. All of the working examples use such techniques. I was unable to identify any working example in the patent that did not use the techniques for targeted loci selection and primer design that are disclosed

in the specification. If the inventors were in possession of techniques for carrying out large scale multiplex PCR that would generate usable nucleic acid for high throughput sequencing and that did not involve loci/primer selection techniques, they would have disclosed such techniques and would have provided a working example

425. As to the '035 patent specifically, the level of written description support is particularly thin in my opinion. The concept of a “universal tail adaptor” is never mentioned in the entirety of the specification. I could not identify any description of a “universal tail adaptor” being used in the context of an embodiment that corresponds to the claims.

426. In view of this, there is unsurprisingly no disclosure in the '454 and '035 patents of a method for carrying out the claimed multiplex PCR without the primer design approaches. Therefore, it is my opinion that '454 and '035 patents are invalid because the claims lack written description support for multiplex PCR amplification that does not utilize any techniques for selection of primers to avoid primer side products, such as primer dimers.

XIV. NATERA'S SIGNATERA™ ASSAY

427. I understand that Dr. Metzker is of the opinion that Signatera™ practices the '454 and '035 patents. D.I. 13 ¶ 124.

A. Signatera™ is Not Shown to Practice the '454 Patent

428. Dr. Metzker sets forth the basis for Signatera™ practicing claim 1 of the '454 patent relying on various exhibits he identifies as disclosing or teaching elements of the claim. *Id.* ¶¶ 124-131.

429. Having considered the cited evidence, I disagree that Dr. Metzker has set forth a basis for Signatera™ practicing claim 1 of the '454 patent. For example, Dr. Metzker fails to

establish the “amplicons having a length of 50-150 bases” in paragraphs 127 to 129, in which he addresses:

Claim 1[b]: performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume; and

Id. ¶¶ 127-129. Dr. Metzker relies on Signatera White Paper (D.I. 13-17), Coombes (2019) (D.I. 13-19), Christensen (2019) (D.I. 13-21), Kotani (2023) (D.I. 13-22), Reinert (D.I. 13-18), and Kirkizlar (2015) (D.I. 13-2). D.I. 13 ¶¶ 127-129. Paragraph 127 of Dr. Metzker’s declaration is silent as to amplicon length. D.I. 13 ¶ 127 (citing D.I. 13-17 at 2).

430. Paragraph 128 of Dr. Metzker’s declaration states:

Coombes (2019), Christensen (2019), and Kotani (2023), which all used Signatera™, illustrate the presence of this claim limitation in Signatera™. For example, Coombes (2019), Christensen (2019), and Kotani (2023) performed targeted multiplex amplification in the same reaction volume to amplify 16 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample and sequencing the amplicons to generate sequence reads having a length of 50 bases.¹⁶⁴

¹⁶⁴ See Coombes (2019) at Abstract, 4256-4258, 4261, Figure 1 and legend, Figure 2 and legend, Supplementary Data at 1-2; see also Christensen (2019) at 1548-1549, Figure 4 and legend, Data Supplement at 3; Kotani (2023) at Methods, citing to Reinert (2019); see Reinert (2019) at eMethods 6.

D.I. 17 ¶ 128. Having reviewed the cited exhibits carefully, I determine that Coombes (2019) discloses that “[s]equencing was performed on an Illumini HiSeq 2500 Rapid Run with 50 cycles of paired-end reads using the Illumina Paired End v2 kit with an average read depth of >100,000 per amplicon.” D.I. 13-19 at 11-12 (Coombes (2019) at Supplementary Data at 1-2).

431. It is my opinion that Coombes (2019)’s disclosure of 50 cycles of paired-end reads does not establish a basis for Signatera employing “amplicons having a length of 50-150 bases” because the number of cycles of paired-end reads is a measure of how many sequencing cycles are conducted to generate sequence reads, and not of the length of the amplicon that is being sequenced. An amplicon being subjected to sequencing could be shorter than fifty bases or longer than 150 bases. Further, Dr. Metzker provides no explanation or reasoning relating to amplicon length. D.I. 17 ¶ 128.

432. The other cited portions of Coombes (2018) are also silent as to the length of amplicons. D.I.13-19 at 2 , 3-5, 11-12 (Coombes (2019) at Abstract, 4256-4258, 4261, Figure 1 and legend, Figure 2 and legend, Supplementary Data at 1-2).

433. Christensen (2019), likewise, discloses that “[s]equencing was performed on an Illumina HiSeq 2500 Rapid Run with 50 cycles of paired-end reads using the Illumina Paired End v2 kit” (D.I. 13-21 at 16 (Data Supplement at 3)), with the other cited portions also being silent as to the length of amplicons (D.I. 13-21 at 3-4, 8 (Christensen (2019) at 1548-1549, Figure 4 and legend).

434. Kotani (2023) and Reinert (2019) are similarly limited, with Reinert (2019) eMethods 6 having the same disclosure that “[s]equencing was performed on an Illumina HiSeq 2500 Rapid Run with 50 cycles of paired-end reads using the Illumina Paired End v2 kit,” with the other cited portions also being silent as to the length of amplicons. D.I. 13-22 (Kotani (2023)); D.I. 13-18 (Reinert (2019)). Also, D.I. 13-18, as filed by Natera, does not include eMethods 6. D.I. 13-18; Ex. 59 (Reinert eMethods).

435. Paragraph 129 of Dr. Metzker’s declaration states:

Kirkizlar (2015) explains that Signatera employs multiplex PCR in a single reaction volume to amplify a bespoke set of targets identified from a patient's tumor sample.¹⁶⁵ The bespoke amplification products from the multiplex PCR “were designed to have a maximum amplicon length of 75 bp” (*i.e.*, 75 bases).¹⁶⁶

¹⁶⁵ See Kirkizlar (2015) at 409.

¹⁶⁶ *Id.*

D.I. 17 ¶ 129.

436. It is my opinion that Kirkizlar does not establish a basis for Signatera employing “amplicons having a length of 50-150 bases” because there is no showing that what is described is Signatera, and I disagree with Dr. Metzker’s opinion that Kirkizlar (2015) provides information about Signatera. *Id.* ¶ 129. First, the multiplex PCR in Kirkizlar differs dramatically from what is employed in Signatera where Natera and Dr. Metzker repeatedly describe Signatera as employing a 16-plex PCR reaction. *Id.* 17 ¶¶ 119, 121; D.I. 13-17 at 2. In contrast to Signatera, in Kirkizlar, the PCR assay is “massively multiplexed PCR” (mmPCR) in which “3168 SNPs were amplified using one primer pair for each SNP.” D.I. 13-2 at 4 (Kirkizlar at 409). In my opinion, such a dramatic difference in multiplex alone establishes that the mmPCR disclosed is not Signatera. Second, Kirkizlar was published in October 2015, while Natera did not launch Signatera until 2017. *Id.* at 2 (indicating publication in 2015: “© 2015 Published by Elsevier Inc. on behalf of Neoplasia Press, Inc.”); D.I. 1 ¶ 17 (“Building on these innovations, in 2017, Natera launched...Signatera®”). This is also contrary to the disclosed assay using mmPCR being Signatera. Third, having reviewed Kirkizlar carefully, there is no mention of Signatera in Kirkizlar. D.I. 13-2. This is also contrary to the disclosed assay using mmPCR being Signatera. In sum, it is my opinion that what Kirkizlar discloses is not Signatera.

437. It is further my opinion, that because Kirkizlar does not disclose Signatera, but rather a different assay using mmPCR, Kirkizlar provides no basis for Signatera employing “amplicons having a length of 50-150 bases.”

438. Accordingly, with no basis provided for Signatera employing “amplicons having a length of 50-150 base,” as required by claim 1, it is my opinion that it is not shown that SignateraTM practices the ’454 patent.

B. SignateraTM is Not Shown to Practice the ’035 Patent

439. Dr. Metzker sets forth the basis for SignateraTM practicing claim 1 of the ’035 patent relying on various exhibits he identifies as disclosing or teaching elements of the claim. D.I. 17 ¶¶ 132-137.

440. Having considered the cited evidence, I disagree that Dr. Metzker has set forth a basis for SignateraTM practicing claim 1 of the ’035 patent. For example, for Claim 1[b]: “amplifying the tagged products..., wherein one of the amplifying steps introduces a barcode and one or more sequencing tags,” Dr. Metzker again relies on Kirkizlar:

In Signatera, one or more sequencing tags are added during one of the amplification steps that also introduces the barcode in the SignateraTM assay. Kirkizlar (2015) explains that Signatera employs multiplex PCR in a single reaction volume to amplify a bespoke set of targets identified from a patient’s tumor sample.¹⁸⁶ The bespoke amplification products are further amplified by a barcoding PCR reaction, which adds “sequencing tags and index sequences,” resulting in final barcoded products.¹⁸⁷

¹⁸⁶ See Kirkizlar (2015) at 409.

¹⁸⁷ *Id.*

Id. ¶ 136 (citing 13-2 at 4 (Kirkizlar at 409). As discussed above, however, there is no showing that what Kirkizlar describes is Signatera, and I disagree with Dr. Metzker’s opinion that Kirkizlar (2015) provides information about Signatera.

441. It is further my opinion, that because Kirkizlar does not disclose Signatera, Kirkizlar provides no basis for Signatera “amplifying the tagged products..., wherein one of the amplifying steps introduces a barcode and one or more sequencing tags.”

442. Accordingly, with no basis provided for Signatera “amplifying the tagged products..., wherein one of the amplifying steps introduces a barcode and one or more sequencing tags,” as required by claim 1, it is my opinion that it is not shown that SignateraTM practices the '035 patent.

I declare under penalty of perjury the laws of the United States of America that the foregoing is true and correct.

Date: October 18, 2023

A handwritten signature in black ink, appearing to read "Brian Van Ness". The signature is written in a cursive, flowing style.

Dr. Brian Van Ness

Exhibit 6

In the Matter Of:

NATERA v

NEOGENOMICS LABORATORIES, INC.

MICHAEL METZKER, PH.D.

September 26, 2023



IN THE UNITED STATES DISTRICT COURT
FOR THE MIDDLE DISTRICT OF NORTH CAROLINA

NATERA, INC.,)
)
Plaintiff,)
)
vs.) Case No. 1:23-cv-629
)
NEOGENOMICS LABORATORIES,)
INC.,)
)
Defendant.)
_____)

VIDEOTAPED DEPOSITION OF

MICHAEL L. METZKER, PH.D.

TUESDAY, SEPTEMBER 26, 2023, 9:25 A.M.

LOS ANGELES, CALIFORNIA

STENOGRAPHICALLY REPORTED BY:
CHERYL HAAB SCOTT, RDR, CRR, CCRR
CA CSR No. 13600
WA CCR No. 3499
NV CCR No. 1003

<p style="text-align: right;">Page 82</p> <p>1 Q In paragraph 108, where you refer to the</p> <p>2 barcoding PCR, you don't actually refer to the</p> <p>3 barcode or the sequencing tag. You, in fact, only</p> <p>4 refer to the universal tail adaptor that you think</p> <p>5 is there; right?</p> <p>6 A Well, Claim 12 is a depending claim; and</p> <p>7 it's a wherein one or more of the universal tail</p> <p>8 adaptors comprises a first universal tail adaptor or</p> <p>9 a second universal tail adaptor. That's the</p> <p>10 limitation. And in my opinion, a personal of</p> <p>11 ordinary skill in the art would have understood the</p> <p>12 adaptors that come in through barcoding are -- can</p> <p>13 meet that limitation.</p> <p>14 DR. SRINIVASAN: Counsel, I'd like to ask</p> <p>15 when you ask the witness questions, please don't</p> <p>16 laugh or demean him.</p> <p>17 DR. WALTER: Okay.</p> <p>18 BY DR. WALTER:</p> <p>19 Q Paragraph 1A -- Claim Element 1A is the one</p> <p>20 that refers to the universal tail adaptor; right?</p> <p>21 A Claim 1A is tagging isolated cell-free DNA</p> <p>22 with one or more universal tail adaptors to generate</p> <p>23 a tail product.</p> <p>24 Q And by virtue of your opinions in Claim 12,</p> <p>25 you rely upon the barcoding PCR process for that</p>	<p style="text-align: right;">Page 84</p> <p>1 DR. SRINIVASAN: Objection. Vague.</p> <p>2 THE WITNESS: Well, I would disagree with</p> <p>3 that.</p> <p>4 BY DR. WALTER:</p> <p>5 Q Which other step besides 1A actually refers</p> <p>6 to a universal tail adaptor?</p> <p>7 A Well, Claim 1B which is amplifying the</p> <p>8 tagged products; so, therefore, they've already</p> <p>9 included the universal tail adaptors.</p> <p>10 Also has a wherein clause:</p> <p>11 "One of the amplifying steps introduces</p> <p>12 barcodes or one or more sequence tags, and those are</p> <p>13 introduced by universal tail adaptors." So I think</p> <p>14 it completely depends on Claim 1B as well.</p> <p>15 Q Claim Step 1B does not include the language</p> <p>16 "universal tail adaptor"; right?</p> <p>17 A It does not. It includes the tagged</p> <p>18 products which incorporated the universal tail</p> <p>19 adaptor in step 1A.</p> <p>20 (Discussion off the record.)</p> <p>21 (Defendant's Exhibit 6 was marked.)</p> <p>22 BY DR. WALTER:</p> <p>23 Q All right. I've handed you a document</p> <p>24 that's been marked as Exhibit 6, but it's entitled</p> <p>25 "Encyclopedia of Medical Devices Instrumentation."</p>
<p style="text-align: right;">Page 83</p> <p>1 claim element; correct?</p> <p>2 A Not necessarily, no.</p> <p>3 Q Your opinion states:</p> <p>4 "According to defendant, Gale (2018) uses</p> <p>5 the eTAm-Seq technology that employs barcoding PCR</p> <p>6 using universal tail adaptors." That's what your</p> <p>7 report says; right?</p> <p>8 A Well, it does say that; but you're not</p> <p>9 focused on the sentence before that that says</p> <p>10 "Gale (2018) described the InVision liquid biopsy</p> <p>11 platform technology (used in the RaDaR assay), which</p> <p>12 includes tagging isolated cell-free DNA with one or</p> <p>13 more universal tail adaptors to generate tagged</p> <p>14 products." I also rely on that sentence.</p> <p>15 Q And then the very next sentence refers to</p> <p>16 the barcoding PCR; right?</p> <p>17 A Well, it does. As I testified, I do believe</p> <p>18 that one -- one of ordinary skill in the art would</p> <p>19 have understood that the one or more universal tail</p> <p>20 adaptors would include the barcoded adaptors used in</p> <p>21 the barcoding PCR step.</p> <p>22 Q And it's only step 1A of the claims that</p> <p>23 refers to the universal tail adaptor. There's no</p> <p>24 other step that refers to the universal tail adaptor</p> <p>25 other than step 1; right?</p>	<p style="text-align: right;">Page 85</p> <p>1 Do you recognize this?</p> <p>2 A I do.</p> <p>3 Q What is this?</p> <p>4 A Well, it's a chapter that I wrote for the</p> <p>5 Encyclopedia of Medical Devices and Instrumentation,</p> <p>6 17, 18 years ago.</p> <p>7 Q It was in 2006, it was published?</p> <p>8 A Correct.</p> <p>9 Q Now, you state on page 384, you have a</p> <p>10 paragraph refers to multiplex PCR.</p> <p>11 Do you see that?</p> <p>12 A 384, I do not see multiplex PCR.</p> <p>13 Q All right. About halfway down on the</p> <p>14 left-hand column, there's a paragraph that begins</p> <p>15 with "multiplex PCR."</p> <p>16 A I do see that paragraph.</p> <p>17 Q And the last sentence reads:</p> <p>18 "Moreover, up to 46 primer pairs have been</p> <p>19 simultaneously amplified by multiplex PCR with</p> <p>20 excellent success (90%) for the large-scale</p> <p>21 identification of human single nucleotide</p> <p>22 polymorphisms by hybridization to high-density DNA</p> <p>23 CHIP arrays."</p> <p>24 Do you see that?</p> <p>25 A I do see that sentence.</p>

<p style="text-align: right;">Page 86</p> <p>1 Q And it was true, in fact, that as of 2009, 2 it had been reported to do over 9,000 targets in a 3 single multiplex reaction for sequencing; correct? 4 DR. SRINIVASAN: Objection. Lacks 5 foundation. Outside the scope. 6 THE WITNESS: I think you're referring to a 7 Fluidigm patent that I've previously relied on, and 8 I believe they reported up to 9,200 targets in a 9 multiplex PCR. 10 BY DR. WALTER: 11 Q For sequencing; correct? 12 A For sequencing. But it's a Fluidigm, and so 13 I don't remember -- I don't think it was actually 14 multiplex PCR. It would have been the Singleton 15 approach that we've seen in Forshew. 16 Q And what was the Singleton approach? 17 A Where a sample would be allocated into 18 individual compartment wells, and then individual 19 primer pairs would be used to amplify specific 20 target regions. 21 DR. WALTER: All right. I've marked as 22 Exhibit 7, Michael Metzker in the matter of Illumina 23 versus Natera in a deposition transcript from 24 May 29th, 2020. 25 (Defendant's Exhibit 7 was marked.)</p>	<p style="text-align: right;">Page 88</p> <p>1 single-tube multiplex PCR of over 9,000 targets for 2 sequencing; correct? 3 A It does have a preamplification step that 4 does target at multiplex PCR. 5 Q Of over 9,000 targets; correct? 6 A That's what they report. 7 DR. WALTER: All right. Do you want to take 8 a break for the lunch? 9 DR. HABERNY: Sure. 10 THE VIDEOGRAPHER: We're going off the 11 record. The time is 12:06 p.m. 12 (Recess.) 13 THE VIDEOGRAPHER: We're back on the record. 14 The time is 12:16 p.m. 15 BY DR. WALTER: 16 Q Okay. Is it accurate that for the '035 17 patent you rely on both -- strike that. 18 Is it accurate that for the '035 patent, you 19 rely upon the preamplification step of 20 Forshew (2012) for both Claim Element 1A and Claim 21 Element 1B? 22 A Yes. 23 Q You rely upon the same amplification process 24 for both elements; correct? 25 A No.</p>
<p style="text-align: right;">Page 87</p> <p>1 BY DR. WALTER: 2 Q You were truthful during this deposition? 3 A I absolutely was, but the date on the front 4 page isn't correct. 5 Q What is the actual date? 6 A It should be April 29th, 2020. 7 Q Okay. 8 A And that's set forth in the testimony. 9 Q Let's take a look at page 188, lines 12 to 10 15. 11 It states: "It is the -- are 9,216 12 different target nucleic acid all amplified in the 13 same reaction." 14 Your testimony was: "Correct." 15 "In the preamplification" -- excuse me -- 16 "in the pre-preamplification, that is a single tube 17 of multiplex PCR reaction." 18 Do you see that? 19 A I do see that. 20 Q Okay. That was your testimony at the time? 21 A It was. And the step I was referring to is 22 the next step where they are allocated into 23 individual wells and amplified separately. 24 Q Okay. So in the PCR -- in the Fluidigm 25 reference that you opined upon, that is teaching a</p>	<p style="text-align: right;">Page 89</p> <p>1 Q All right. Please explain. 2 A Well, I am relying on the preamplification 3 step for the element in Claim 1A of tagging isolated 4 cell-free DNA with one or more universal tail 5 adaptors to generate tagged products. I am also 6 relying on the preamplification step. In 1B, where 7 the amplifying the tagged products one or more times 8 to generate final amplification products, depend on 9 the wherein, one of the amplification steps 10 comprises target amplification of a plurality of 11 single nucleotide polymorphisms. But I'm also 12 relying on the second step where the -- and I'm 13 looking at Forshew figure -- Forshew (2012), the 14 figure in paragraph 109 where it says "sequence 15 adaptor and barcode attachment." I'm relying on 16 that PCR step meeting the claim element wherein one 17 or more of the amplification steps introduce as a 18 barcode in one or more sequencing tags. 19 Q You understand that it's the -- your 20 contention is it's the preamplification step that 21 generates the tagged products that are referred to 22 in Claim Element 1A of the '035 patent; correct? 23 A The preamplification step is creating the 24 tagged products in Claim 1A of the '035 patent. 25 Q And then you rely upon the preamplification</p>

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<p>1 process for subsequent step 1B that refers to</p> <p>2 amplifying the tagged products wherein one of the</p> <p>3 amplification steps comprises a targeted</p> <p>4 amplification; correct?</p> <p>5 A That is correct.</p> <p>6 Q Okay. Is it accurate, then, that you're</p> <p>7 taking the preamplification step and breaking it</p> <p>8 into two parts: One part that you allocate to step</p> <p>9 1A, and then another part that you allocate to step</p> <p>10 1B?</p> <p>11 DR. SRINIVASAN: Objection. Vague.</p> <p>12 THE WITNESS: I am not breaking apart the</p> <p>13 preamplification step.</p> <p>14 BY DR. WALTER:</p> <p>15 Q You are relying upon the entirety of the</p> <p>16 preamplification step for both step 1A to generate</p> <p>17 the tagged products and also Claim Step 1B that</p> <p>18 refers to amplifying the tagged products one or more</p> <p>19 times wherein one of the amplification steps</p> <p>20 comprises targeted amplification; is that right?</p> <p>21 DR. SRINIVASAN: Objection. Vague.</p> <p>22 THE WITNESS: I'm relying on the</p> <p>23 preamplification step, which includes 15 cycles. I</p> <p>24 explained that, actually, in my review article that</p> <p>25 you've presented as figure -- or Exhibit 7, where</p>	<p>1 report, do you explain that the -- well -- let's do</p> <p>2 it this way.</p> <p>3 For Claim Element 1A of the process of</p> <p>4 generating the tagged products, you are relying upon</p> <p>5 the first cycle of the PCR preamplification;</p> <p>6 correct?</p> <p>7 A Yes, that would be the first time the</p> <p>8 target-specific primers will hybridize to the</p> <p>9 cell-free DNA.</p> <p>10 Q And then for step 1B, you're relying upon</p> <p>11 the subsequent 14 cycles; correct?</p> <p>12 A Well, I'm only relying on those cycles that</p> <p>13 amplify the tagged products.</p> <p>14 Q Where, if anywhere in your report, do you</p> <p>15 state that you're only relying upon the first cycle</p> <p>16 for step 1A and then the subsequent 14 cycles that</p> <p>17 only amplify the tagged products for step 1B?</p> <p>18 A Well, I generally explain it in -- first, I</p> <p>19 can have a chance to look at paragraph 87.</p> <p>20 So on page 47, the first sentence says:</p> <p>21 "Gale (2018) describes the InVision liquid</p> <p>22 biopsy platform technology (used in the RaDaR assay)</p> <p>23 which includes tagging isolated cell-free DNA with</p> <p>24 one or more universal tail adaptors to generate</p> <p>25 tagged products."</p>
Page 91	Page 93
<p>1 the initial PCR amplifies the genomic targets, but</p> <p>2 subsequent rounds of PCR begin to amplify the</p> <p>3 product from the last cycle; and it's that product</p> <p>4 from the last cycle that now constitutes the tagged</p> <p>5 products that are being amplified. So that's all</p> <p>6 happening within that preamplification step.</p> <p>7 BY DR. WALTER:</p> <p>8 Q So you allocate a portion of that</p> <p>9 preamplification step to the tag and process</p> <p>10 generation, and then another portion of it to the</p> <p>11 amplifying the tagged products; is that right?</p> <p>12 A No. I'm not allocating anything. I'm just</p> <p>13 describing the PCR cycle. And it is illustrated in</p> <p>14 figure 1 of Exhibit 7, where the first cycle of PCR</p> <p>15 amplifies from the target sequence. But subsequent</p> <p>16 cycles of primer extension, template denaturation,</p> <p>17 and then hybridization -- all of the products now</p> <p>18 that are being amplified are from the previous</p> <p>19 cycle. And, in this case, it's the tagged products.</p> <p>20 Q Now, figure 1 of Exhibit 7, that's not in</p> <p>21 your report anywhere?</p> <p>22 A It is not. It's one of the references that</p> <p>23 is cited in my CV, and I do rely on my knowledge in</p> <p>24 providing opinions in this declaration.</p> <p>25 Q Okay. Now where, if anywhere in your</p>	<p>1 A person of ordinary skill in the art would</p> <p>2 understand how PCR works; and in subsequent rounds,</p> <p>3 the products of the previous round become the</p> <p>4 template for the next cycle.</p> <p>5 Q Okay. Where do you say anywhere in your</p> <p>6 report that you're relying upon the first cycle of</p> <p>7 the PCR to generate the tagged products, and where</p> <p>8 do you point out that first cycle?</p> <p>9 A Well, I don't explicitly point out the first</p> <p>10 cycle. It's well known how the PCR cycle works.</p> <p>11 Q Okay. Now, where, if anywhere, do you state</p> <p>12 in your report that you're relying upon the</p> <p>13 subsequent 14 cycles that only amplify the tagged</p> <p>14 products? Where do you say that? Where do you</p> <p>15 point out those 14 cycles?</p> <p>16 A Well, I'm looking at paragraph 94, and I</p> <p>17 say:</p> <p>18 "The accused assay satisfies this claim</p> <p>19 limitation. For example, the RaDaR assay includes</p> <p>20 amplifying the tagged products one or more times to</p> <p>21 generate final amplification products wherein one of</p> <p>22 the amplification steps comprises target -- targeted</p> <p>23 amplification of a plurality of single nucleotide</p> <p>24 polymorphism (SNP loci) in a single reaction</p> <p>25 volume."</p>

Exhibit 10

IN THE UNITED STATES DISTRICT COURT
FOR THE DISTRICT OF DELAWARE

CAREDX, INC. and THE BOARD OF)
TRUSTEES OF THE LELAND)
STANFORD JUNIOR UNIVERSITY,)
)
Plaintiffs,)
)
v.) C.A. No. 19-567 (CFC) (CJB)
) CONSOLIDATED
NATERA, INC.,)
)
Defendant.)

**NATERA INC.’S OPENING BRIEF IN SUPPORT OF ITS MOTION FOR
SUMMARY JUDGMENT UNDER 35 U.S.C. § 101**

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June 11, 2020

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Rules and Regulations

Fed. R. Civ. P. 56 1

GLOSSARY OF TERMS

TERM	DEFINITION
the Patents	Collectively, U.S. Patent Nos. 8,703,652; 9,845,497; and 10,329,607
the '652 patent	U.S. Patent No. 8,703,652
the '497 patent	U.S. Patent No. 9,845,497
the '607 patent	U.S. Patent No. 10,329,607
the Claims	The asserted claims of the Patents: '652 patent: claims 1-3, 6, 11-12, 14-15 '497 patent: claims 1-6, 9-10, 12, 15, 17, 19-21, 23, 25, 27 '607: all claims
the Patentees	The named inventors on the face of the Patents.
CareDx	Collectively, plaintiffs CareDx, Inc. and The Board of Trustees of the Leland Stanford Junior University
Natera	Defendant Natera, Inc.
FAC	First Amended Complaint (D.I. 74)
DNA	Deoxyribonucleic Acid
cfDNA	Cell Free DNA
UF-#	Uncontested Statement of Facts
A-¶#	Appendix A, Declaration of Professor John A. Quackenbush, Ph.D.
B#	Appendix of Exhibits

I. NATURE AND STAGE OF THE PROCEEDINGS

On March 6, 2019, CareDx filed a Complaint against Natera (D.I. 1) alleging that Prospera, Natera's Kidney Transplant Rejection Test, infringes the '652 patent and the '497 patent. On March 12, 2020, CareDx filed the FAC and added the '607 patent to the case. D.I. 74.¹ At the Court's invitation (Apr. 30, 2020 Oral Order), Natera brings this motion for summary judgment pursuant to Federal Rule of Civil Procedure 56 and requests an order that the Claims are invalid because they are unpatentable under 35 U.S.C. § 101.

II. SUMMARY OF THE ARGUMENT

The Claims are unpatentable under 35 U.S.C. § 101 because they are directed to detecting an undisputedly natural phenomenon using techniques the patentee concedes are conventional. The natural phenomenon is the presence of foreign genetic material—donor-specific cell-free nucleic acid or cfDNA—that is

¹ Natera previously moved to dismiss the Complaint and FAC on the grounds that the Patents are invalid under 35 U.S.C. § 101. The parties' briefing and Magistrate Judge Burke's Report and Recommendation ("RR") on Natera's motion to dismiss the initial Complaint are filed at D.I. 9, 10, 15, 19, 53, 63, and 68. After Judge Burke issued his RR, CareDx filed the FAC. D.I. 74. While Natera's motion to dismiss the initial Complaint was pending, the Court vacated the RR and denied Natera's motion to dismiss as moot. Natera thereafter moved to dismiss the FAC (D.I. 86, 87) but then withdrew the motion. D.I. 97. Upon withdrawing Natera's motion to dismiss the FAC, the Court stayed the case and scheduled early summary judgment briefing on patentability under 35 U.S.C. § 101. *See* Apr. 30, 2020 Oral Order.

released from a transplanted organ into a transplant recipient's body.² The conventionality of the claimed techniques used to detect this cfDNA cannot be disputed. The Patents' written description admits "[t]he practice of the present invention employs, unless otherwise indicated, *conventional techniques* ... which are within the skill of the art."³ Nowhere does the written description or claims "otherwise indicate" that the claimed techniques are anything other than conventional. And the prior and contemporaneous scientific literature from 2009 and before confirms the same.

The conventional techniques are:

- (i) "obtaining" a "sample" from the recipient that contains cfDNA;
- (ii) "genotyping" the transplant donor and/or recipient to develop genetic profiles (or "genotypes");
- (iii) "sequencing" the cfDNA from the sample using "multiplex" or "high-throughput" sequencing; and
- (iv) "determining" / "quantifying" the amount of donor cfDNA.

These techniques have been and are used to detect the natural differences ("polymorphisms") between a donor's cfDNA and a recipient's cfDNA. But the Patents do not describe any non-conventional practice of these techniques or any

² The '652 patent is directed to the related but equally natural correlation between donor-specific cfDNA and the likelihood that the transplanted organ is failing or being rejected.

³ UF-10; B0012 at 5:36-48. All emphasis in this Motion is added unless otherwise noted.

improvement or modification of them. The Patents' written description admits these techniques—taken individually or together as a whole—were routinely practiced by 2009 using commercially available equipment.

The scientific literature also shows the claimed techniques were routinely used before November 2009. For example, the claimed techniques were used to detect cell-free nucleic acids from analogous natural phenomena such as fetal cfDNA in a pregnant woman's blood, tumor cfDNA in a cancer patient's blood, and bacterial or viral cfDNA in an infected person's blood. Both the Patentees and numerous other scientists realized that the claimed techniques could be applied to detect any of these natural phenomena because they share the same fundamental feature: the presence of cfDNA in a patient's blood from different sources with different polymorphisms and different genotypes.

Accordingly, the Patents here claim detecting yet another naturally occurring example of this phenomenon in the transplant context—a context that many scientists before 2009 recognized as a suitable application for these methods. That is the only distinction from the prior art that CareDx identifies. But that distinction is not enough to survive summary judgment. Claiming conventional methods to predictably detect a different (but analogous) natural phenomenon does not render the Claims patentable.

Further, the U.S. Supreme Court and Federal Circuit opinions in every

analogous case confirm the Claims are unpatentable. In those cases, the courts invalidated claims directed to detecting or correlating natural relationships between:

- i. naturally metabolized drugs and safe treatment therewith (*Mayo Collaborative Servs. v. Prometheus Labs., Inc.*, 566 U.S. 66 (2012));
- ii. naturally inherited paternal genes and naturally occurring fetal cfDNA in a pregnant mother's blood (*Ariosa Diagnostics, Inc. v. Sequenom, Inc.*, 788 F.3d 1371 (Fed. Cir. 2015));
- iii. coding and non-coding sequences in the human genome (*Genetic Techs. Ltd. v. Merial L.L.C.*, 818 F.3d 1369 (Fed. Cir. 2016));
- iv. natural inflammatory enzymes and cardiovascular disease (*Cleveland Clinic Found. v. True Health Diagnostics LLC*, 859 F.3d 1352 (Fed. Cir. 2017)); and
- v. naturally occurring autoimmune antibodies and autoimmune disease (*Athena Diagnostics, Inc. v. Mayo Collaborative Servs., LLC*, 915 F.3d 743 (Fed. Cir. 2019)).

Because there is no material fact in dispute, summary judgment holding the Claims unpatentable under 35 U.S.C. § 101 is warranted.

III. STATEMENT OF FACTS

A. Technological Background

1. The Structure Of Nucleic Acids

Nucleic acids, such as the well-known DNA and RNA, are present in all living things and encode information needed for cellular growth and function. A-

¶39. While there are many types of nucleic acids, DNA⁴ is the most relevant nucleic acid to the Patents. A-¶39. DNA is a double-stranded nucleic acid, and each strand is made up of a sequence of naturally occurring nucleotide bases. A-¶40. There are four possible bases that make up DNA—adenine (“A”), cytosine (“C”), guanine (“G”), and thymine (“T”). A-¶40. The sequence of bases on the strands is unique to every individual (except identical twins). A-¶43.

2. Genes, Genotypes, And Polymorphisms

Certain sequences of DNA nucleotides comprise genes that provide a code or blueprint for producing other molecules (such as proteins) enabling cell function. A-¶42. All DNA is normally located on chromosomes (threads of nucleic acid and protein) that are stored in the nuclei of cells. A-¶41. A specific nucleotide sequence that codes for a particular trait in individuals is called a “genotype,” and the sum of an individual’s DNA is called a “genome.” A-¶43. There are naturally occurring differences in genotypes, called “polymorphisms,” that make each individual’s genotype unique. A-¶43. One type of polymorphism is a “single nucleotide polymorphism” (“SNP”), wherein a different nucleotide base is present at a single position (“locus”) in different individuals. A-¶43.

3. Zygoty: Homozygoty And Heterozygoty

Every human normally has two copies of each chromosome, one inherited

⁴ The pertinent properties of nucleic acids, such as DNA, are well-understood. See *Ass’n for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. 576, 582 (2013).

from its mother and another from its father. A-¶44. “Zygosity” describes the similarity or difference between maternally and paternally inherited chromosomes at any given locus. A-¶44. An individual is “homozygous” for a particular locus if it has the same sequence on both its maternally inherited and paternally inherited chromosomes at that locus. A-¶44. An organism is “heterozygous” for a particular locus if the sequence on its maternally inherited chromosome differs from that of its paternally inherited chromosome at that locus. A-¶44.

4. Cell-Free DNA (“cfDNA”)

DNA is typically found inside cells, but when cells die (via a process called “apoptosis”) their DNA is broken up into small fragments and released into circulating bodily fluids as “cell-free DNA” or “cfDNA.” UF-4; A-¶47. The existence of cfDNA was discovered as early as 1948. UF-3. Since that time, cfDNA has been studied in various contexts, including cancer, pregnancy, infectious disease, and transplant. UF-3-7; A-¶¶50-56.

5. cfDNA Genotypes In The Transplant, Prenatal, Infectious Disease And Cancer Contexts

In a variety of situations, cells in a patient having DNA that is different from most of a patient’s own DNA may die and release their unique genetic material into the patient as cfDNA. UF-4; A-¶49. For example, tumor cells in a patient naturally die and release abnormally mutated cfDNA into the patient’s blood. UF-6; A-¶54. These mutations are unique to the tumor and render its cfDNA distinct

from the patient's normal cells. UF-6; A-¶¶54-55. Similarly, in pregnant women, cells from the fetus or placenta will naturally die and release the fetus' unique cfDNA into the mother's blood. UF-5; A-¶¶52-53. The same is true in patients with an infectious disease—bacterial or viral agents will naturally die and release their unique cfDNA into the blood of an infected patient. UF-4; A-¶56. Organ transplants are no different. UF-7; A-¶¶50-51. The cells of a transplanted donor organ will naturally die and release the donor's unique cfDNA into the transplant recipient's blood. UF-7; A-¶50. In each of these cases, the genotype of the foreign cfDNA (tumor, fetus, virus/bacteria, donor) will differ from the genotype of the patient's own cfDNA (which also is regularly released into the patient's blood). UF-5-7; A-¶57; *e.g.*, B0013 at 7:37-46. And in each case, routine and conventional methods for detecting differences in the genotypes of the cfDNA can be used to determine their different sources. UF-5-7; A-¶57.

In the case of organ transplants, the body of the recipient may reject the transplanted organ, or the organ may fail for some other reason. UF-7; A-¶51. In the case of rejection, the immune system of the recipient attacks the transplanted organ, causing the organ's cells to die more rapidly than normal. UF-7; A-¶51. In the event of transplant failure, increased rates of cell death in the organ prompt the release of more donor-specific cfDNA into the recipient's circulation than would be expected if the transplanted organ was healthy. UF-7; A-¶51. Thus, heightened

cell death in the transplanted organ corresponds to heightened donor-specific cfDNA in the recipient's circulation. UF-7; A-¶51. As such, detecting high quantities of donor cfDNA indicates a greater likelihood that the transplant is either failing or being rejected. UF-7; A-¶51.⁵

6. Genotyping

Genotyping is a process for determining the genetic make-up of an individual by examining particular portions of that individual's DNA sequences. A-¶43. Common variants of different sequences or genes are called "alleles." A-¶43, 127. Genotyping is the identification of the specific allele or alleles an individual has inherited. A-¶43. By 2009, genotyping—including SNP genotyping—was a well-known, commercially available process for analyzing DNA in a variety of applications. UF-13-15; A-¶¶67, 79-80; B0019 at 20:31-41 ("*[g]enotyping* of the transplant donor and/or the transplant recipient *may be performed by any suitable method known in the art*").⁶

⁵ UF-4, 7; B0013 at 7:41-46 ("[A]s cell-free DNA or RNA often arises from apoptotic cells, the relative amount of donor-specific sequences in circulating nucleic acids should provide a predictive measure of on-coming organ failure in transplant patients for many types of solid organ transplantation including, but not limited to, heart, lung, liver, and kidney.").

⁶ UF-8, 14-15; B0016 at 13:51-61 (explaining that genotyping including SNP genotyping could be performed using "existing genotyping platforms known in the art including the one described herein") (internal citations to scientific literature omitted).

7. Sequencing

Sequencing is the process of determining the exact order of the bases in a DNA fragment. A-¶100. As discussed earlier, sequences can be any ordered combination of the bases Adenine (A), Guanine (G), Cytosine (C), or Thymine (T), and a DNA molecule can be anywhere from a few bases to billions of bases in length. A-¶40. Like genotyping, sequencing was well-known and routinely used before the Patents were filed (UF-16, 19; B0017 at 15:8-16:41), as were methods for simultaneously sequencing multiple strands of DNA in a high-throughput instrument. UF-18-20, 22; A-¶¶100-02. Parallel, high-throughput sequencing also is called multiplex sequencing, and one way to perform multiplex sequencing is via “sequencing-by-synthesis.” UF-22; A-¶101. Sequencing-by-synthesis involves enzymatically creating a DNA strand that is complementary to one being sequenced. A-¶101. This is done by adding one nucleotide at a time while keeping track of each nucleotide after it is added. A-¶101. All of these technologies were known as early as 1993 and have been available commercially since at least 1999. UF-18-20, 22; A-¶¶103-13; B0017 at 15:8-16:41 (describing commercial products for multiplex, high-throughput, and sequencing-by-synthesis).

B. The Claims

The Claims⁷ are all directed to detecting donor-specific cfDNA circulating in a transplant recipient. A-¶¶27-38. In the case of the '652 patent, once this donor-specific cfDNA is detected, its measured quantity is then correlated to possible transplant failure. Claim 1 of the '652 patent recites a “method for detecting transplant rejection . . . or organ failure.” Claim 1 of the '497 patent recites a “method of detecting donor-specific circulating cell-free nucleic acids in a solid organ transplant recipient.” And claim 1 of the '607 patent recites a “method of quantifying kidney transplant-derived circulating cell-free deoxyribonucleic acids in a human kidney transplant recipient.”

Although the Claims use different words at times, and some limitations use more words than others, the Claims nevertheless all recite the same steps for detection and correlation:

- **Obtaining/providing a biological sample containing cfDNA from a transplant recipient**
 - See '652 Patent Claim 1(a) (“providing a sample comprising [cfDNA]”)⁸
 - See '497 Patent Claim 1(c) (“obtaining a biological sample”)⁹
 - See '607 Patent Claims 1(a) and (b) (“providing a plasma sample” and “extracting circulating [cfDNA]”)

⁷ The Patents have a common written description and one independent claim each. UF-1.

⁸ '652 patent claims 3 and 12-16 recite similar sampling and transplant-related limitations. A-¶¶65, 172, 186, 191.

⁹ '497 patent claims 2, 9, 12-14, 27, and 28 recite similar sampling and transplant-related limitations. A-¶¶65, 175, 187, 192.

- **Genotyping the transplant donor and/or recipient to establish profiles of genetic polymorphisms (or SNPs)**
 - See '652 Patent Claim 1(b) (“obtaining a genotype ... to establish a polymorphism profile”)¹⁰
 - See '497 Patent Claims 1(a) and (b) (“genotyping ... to obtain a SNP profile”)¹¹
 - See '607 Patent Claim 1(c) (“performing a selective amplification of [SNPs] ... by [PCR]”); Claim 1(f) (“using markers distinguishable between said [recipient and donor] . . . [that] comprise [SNPs]”)¹²
- **Performing multiplex or high-throughput sequencing of the cfDNA to detect the genotyped polymorphisms (or SNPs)**
 - See '652 Patent Claim 1(c) (“multiplex sequencing of the [cfDNA] in the sample followed by analysis of the sequencing results using the polymorphism profile”)¹³
 - See '497 Patent Claim 1(d) (“determining an amount of donor-specific [cfDNA] by ... high-throughput sequencing or [dPCR]”)¹⁴
 - See '607 Patent Claims 1(d) and (e) (“performing a high throughput sequencing reaction ... compris[ing] ... sequencing-by-synthesis ... [and] ... providing sequences from said high throughput sequencing reaction”)¹⁵

¹⁰ '652 patent claims 2 and 11 recite similar genotyping and polymorphism-related limitations. A-¶¶65, 170, 173.

¹¹ '497 patent claims 6, 15-18, 24, and 25 recite similar genotyping and polymorphism-related limitations. A-¶¶65, 171, 194.

¹² '607 patent claims 2-5 recite similar genotyping and polymorphism-related limitations. A-¶¶65, 172.

¹³ '652 patent claims 4-6, and 10 recite similar sequencing-related limitations. A-¶¶65, 178.

¹⁴ '497 patent claims 3-5, 10, 11, 26, and 30 recite similar sequencing-related limitations. A-¶¶65, 179, 189.

¹⁵ '607 patent claim 6 recites similar sequencing-related limitations. A-¶¶65, 180.

- **Quantifying the transplant cfDNA in the sample by detecting the genetic differences in the sequences**
 - See '652 Patent Claim 1(d) (“determining a quantity of [transplant cfDNA] based on the detection of [donor and recipient cfDNA] by the multiplexed sequencing”)
 - See '497 Patent Claim 1(d) (“determining an amount of [transplant cfDNA] ... by detecting a homozygous or a heterozygous SNP within the [transplant cfDNA]”)
 - See '607 Patent Claim 1(f) (“quantifying an amount of [transplant cfDNA] ... using markers distinguishable between ... recipient and ... donor”).

A-¶29.

IV. LEGAL STANDARD

A. Patentable Subject Matter

“‘[L]aws of nature, natural phenomena, and abstract ideas’ are not patentable.” *Mayo Collaborative Servs. v. Prometheus Labs, Inc.*, 566 U.S. 66, 70 (2012) (citation omitted). Only “innovative” or “inventive” uses of natural phenomena are afforded patent protection. *Ass’n for Molecular Pathology v. Myriad Genetics, Inc.*, 569 U.S. 576, 595 (2013). In determining whether patent claims reciting natural phenomena are eligible for protection under 35 U.S.C. § 101, two questions are asked (*i.e.*, “steps”): (1) whether the patent is directed to the natural phenomenon, and if so, (2) whether the claims recite an inventive concept “sufficient to ensure that the patent in practice amounts to significantly more than a patent upon the natural law itself.” *Mayo*, 566 U.S. at 72-73, 77-80; *Ariosa*

Diagnostics, Inc. v. Sequenom, Inc., 788 F.3d 1371, 1375 (Fed. Cir. 2015). “The inventive concept necessary at step two of the *Mayo/Alice* analysis cannot be furnished by the unpatentable law of nature (or natural phenomenon or abstract idea) itself. That is, under the *Mayo/Alice* framework, a claim directed to a newly discovered law of nature (or natural phenomenon or abstract idea) cannot rely on the novelty of that discovery for the inventive concept necessary for patent eligibility[.]” *Genetic Techs. Ltd. v. Merial L.L.C.*, 818 F.3d 1369, 1376 (Fed. Cir. 2016).

Further, “[t]he prohibition against patenting abstract ideas cannot be circumvented by attempting to limit the use of the formula to a particular technological environment or adding insignificant post solution activity.” *Mayo*, 566 U.S. at 73 (internal quotation marks and citation omitted). Accordingly, “simply appending conventional steps, specified at a high level of generality, to laws of nature, natural phenomena, and abstract ideas cannot make those laws, phenomena, and ideas patentable.” *Id.* at 82.

B. Summary Judgment

“Patent eligibility under 35 U.S.C. § 101 is a question of law.” *Genetic Techs.*, 818 F.3d at 1373. However, whether something is well-understood, routine and conventional is a factual question. *Berkheimer v. HP Inc.*, 881 F.3d 1360, 1369 (Fed. Cir. 2018). Summary judgment is appropriate where there is no genuine

A court need not individually address claims not identified by the non-moving party if the court identifies a representative claim and “all the claims ‘are substantially similar and linked to the same abstract idea.’” *Content Extraction & Transmission LLC v. Wells Fargo Bank, Nat. Ass’n*, 776 F.3d 1343, 1348 (Fed. Cir. 2014) (citations omitted).

The Claims are directed to detecting a natural phenomenon—donor-specific cfDNA from a transplanted organ that circulates in the blood of a transplant recipient. The '652 patent claims are directed to the additional unpatentable concept of correlating the detected quantity of donor-specific cfDNA to transplant health—another natural phenomenon. The Claims perform this detection using conventional techniques—an indisputable fact in view of the Patentees' admissions and the corroborating scientific literature. Settled Supreme Court and Federal Circuit precedent further confirm that the Claims are not patent

eligible,¹⁶ while recent authority upholding the patentability of certain life-sciences patents are inapposite. *See infra* Section V(D).

A. The Claims Are Directed To Natural Phenomena

The Claims fail at Step One of the *Mayo/Alice* inquiry because they are directed to conventionally detecting natural phenomena—the presence of donor-specific cfDNA circulating in a transplant recipient’s body, and in the case of the ’652 patent, the relationship between that donor-specific cfDNA and the health or status of the donor organ. *See BSG Tech LLC v. Buyseasons, Inc.*, 899 F.3d 1281, 1287 (Fed. Cir. 2018) (“For an application of an abstract idea to satisfy step one [of the *Mayo* framework], the claim’s focus must be on something other than the abstract idea itself.”).

To start, the Patents admit that cfDNA occurs naturally as a result of cell death in a transplant recipient’s body. The Patents further describe cell death in a transplanted organ (and the consequent release of donor-specific cfDNA) as something that naturally occurs more frequently when a transplant is failing. UF-7; B0013 at 7:40-46; 8:18-21. That admission cannot be disputed. A-¶63. The single nucleotide polymorphisms (“SNPs”) recited in the Claims for distinguishing between donor-specific and recipient cfDNA also are—again by

¹⁶ *See, e.g., Alice Corp. Pty. Ltd v. CLS Bank Int’l*, 573 U.S. 208, 217 (2014); *Mayo*, 566 U.S. at 77; *Ariosa*, 788 F.3d at 1373-76; *Cleveland Clinic*, 859 F.3d at 1361; *In re BRCA1- & BRCA2-Based Hereditary Cancer Test Patent Litig.*, 774 F.3d 755, 764 (Fed. Cir. 2014).

the Patents’ own admission—naturally occurring genetic differences that were conventionally used prior to the Patents to distinguish different DNA samples to determine their source. UF-8-9; B0012 at 6:61-64; B0013 at 7:40-46. This admission also cannot be disputed. A-¶¶59-66. Detecting those natural phenomena, or correlating the presence of donor-specific cfDNA in a patient’s circulation to that patient’s transplant health, are mere observations of natural phenomena and nothing more. A-¶¶59-66.

The Claims “begin and end” with the very natural phenomena they observe, and are thus “directed to matter that is naturally occurring.” *Ariosa*, 788 F.3d at 1376. They do not create anything new or unnatural, such as new or altered DNA sequences, or unnatural DNA preparations. Instead, the Claims detect what already exists in nature.¹⁷ A-¶¶59-66. For example, claim 1(a) of the ’652 patent starts with “a sample comprising cell-free nucleic acids from a subject who has received a transplant,” and element 1(d) ends with “diagnosing, predicting, or monitoring a transplant status or outcome of the subject ... by determining a quantity of the donor cell-free nucleic acids based on the detection

¹⁷ See, e.g., *Genetic Techs.*, 818 F.3d at 1375-76 (finding that “the claims are directed to matter that is naturally occurring,” and the method resulted in “no creation or alteration of DNA sequences”); cf. *Rapid Litig. Mgmt. Ltd. v. CellzDirect*, 827 F.3d 1042, 1047-50 (Fed Cir. 2016) (claiming new methods for generating a preparation with unnaturally high quantities of fetal cdDNA); *Illumina, Inc. v. Ariosa Diagnostics, Inc.*, 952 F.3d 1367, 1371-74 (Fed. Cir. 2020) (claiming new methods for preparing a select and unnatural population of liver cells).

of the donor cell-free nucleic acids and subject cell-free nucleic acids” in the sample. B0023 at 27:41-43; 27:59-63. In the ’497 patent, the preamble of claim 1 starts with “detecting donor-specific circulating cell-free nucleic acids in a solid organ transplant recipient,” and ends in element 1(d) with “determining an amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample.” B0045 at 28:2-3; 28:24-26. And in the ’607 patent, claim 1(a) begins with “providing a plasma sample from said human kidney transplant recipient” and ends in element 1(e) with “quantifying an amount of said kidney transplant-derived circulating cell-free deoxyribonucleic acid in said plasma sample from said human kidney transplant recipient to obtain a quantified amount.” B0072 at 28:59-60; B0073 at 29:39-42. Accordingly, the Claims are directed to a natural phenomenon and do not pass Step One.¹⁸

B. The Claims Recite Only Standard, Unimproved Techniques For Detecting Or Quantifying Donor-Specific cfDNA

Patent claims that purport to “detect[] a natural law ‘with no meaningful

¹⁸ See, e.g., B0013 at 7:40-46; see *Mayo*, 566 U.S. at 77 (finding the claims ineligible because they “set forth laws of nature—namely, relationships between concentrations of certain metabolites in the blood and the likelihood that a dosage of a [] drug will prove ineffective or cause harm”); *Ariosa*, 788 F.3d at 1373-76 (invalidating claims “directed to detecting the presence of a naturally occurring thing or a natural phenomenon, [cell-free fetal DNA] in maternal plasma or serum”); *Cleveland Clinic*, 859 F.3d at 1361 (finding unpatentable claims detecting enzyme in order to diagnose cardiovascular risk); *23andMe, Inc. v. Ancestry.com DNA, LLC*, 356 F. Supp. 3d 889, 904-06 (N.D. Cal. 2018) (finding unpatentable claims that detected correlation that exists in nature).

non-routine steps” are “directed to” that natural law and are unpatentable. *Athena*, 915 F.3d at 752 (quoting *Cleveland Clinic*, 859 F.3d at 1361). Here, the Claims only recite routine steps, in a routine order, to detect naturally occurring donor-specific cfDNA. A-¶¶67-128. This renders the Claims unpatentable.

1. The Claims As A Whole Recite No Inventive Concept

There is nothing in the Claims as a whole that gives rise to an inventive concept “sufficient to ensure that the patent in practice amounts to *significantly more* than a patent upon the natural law itself.” *Mayo*, 566 U.S. at 73. “To save a patent” under *Alice/Mayo*, “an inventive concept ‘must be evident in the claims.’” *WhitServe LLC v. Dropbox, Inc.*, No. 18-665-CFC, 2019 WL 3342949, at *5 (D. Del. July 25, 2019) (quoting and citing *Two-Way Media Ltd. v. Comcast Cable Commc’ns, LLC*, 874 F.3d 1329, 1338 (Fed. Cir. 2017)). Here, there is nothing evident in the written description or Claims to save them. A-¶¶67-72.

The Patents claim only the application of conventional techniques and previously used combinations for detecting and quantifying cfDNA from multiple sources. *See infra* Section V(B)(2); A-¶¶67-72. In fact, the Patents readily admit there is nothing inventive about the techniques used to practice the Claims: “[t]he practice of the present invention employs, unless otherwise indicated, conventional techniques ... which are within the skill of the art.” B0012 at 5:36-48. The Patents further explain that genotyping and quantitation can be performed by sequencing,

which as explained in detail below, was a conventional technique performed with off-the-shelf commercial devices by 2009:

Genotyping donor and recipient nucleic acids, and/or detection, identification and/or ***quantitation of the donor-specific nucleic acids*** after transplantation (e.g. ***polymorphic markers such as SNPs***) ***can be performed by sequencing*** such as whole genome sequencing or exome sequencing.

B0017 at 15:2-6; A-¶¶78-81. The Patents also acknowledge that its techniques were being used for diagnosing other conditions where DNA sequences that are different from a patient's normal genotype are present in a patient's sample, such as in pregnancy or cancer. B0012-B0013 at 6:57-7:46; A-¶137. The Patents explain:

In all these applications of circulating nucleic acids, the presence of sequences differing from a patient's normal genotype has been used to detect disease. In ***cancer***, mutations of genes are a tell-tale sign of the advance of the disease; in ***fetal diagnostics***, the detection of sequences specific to the fetus compared to maternal DNA allows for analysis of the health of the fetus.

B0013 at 7:30-36 (emphasis added).

Moreover, there is no evidence the Patentees modified the techniques the Patents describe as routine, conventional, and widely used in the cancer and fetal diagnostic fields, and even if they did, the Claims do not contain any such limitations. A-¶¶68-69. In fact, the evidence proves the opposite. Inventors Stephen Quake and Hannah Valentine admitted in a Stanford publication that their

transplant-focused technique was based upon a well-known combination of techniques used to detect fetal cfDNA in a pregnant patient:

The current study began when Valantine noticed research by Quake in 2008 showing that it is possible to detect fetal chromosomal abnormalities by sequencing cell-free DNA fragments in a maternal blood sample.

“When I saw that, I thought, wow, *this technique* could probably be used to monitor heart rejection,” said Valantine, noting that cells damaged during rejection also release DNA into the circulatory system.

B00390; A-¶138. Stanford published the same admission:

The *cell-free DNA technique* hinges on the existence in the genome of *naturally occurring regions* of variation called single nucleotide polymorphisms, or SNPs. In 2008, Hannah Valantine, then a Stanford professor of cardiology, realized that *a DNA-sequencing technique* developed in Quake’s lab to pick out small quantities of fetal DNA from a pregnant woman’s blood might also be useful to track the fate of a transplanted organ.

B0393. If the claimed techniques were being used in analogous fields before the Patents were filed, then their unmodified use in the transplant context cannot be non-conventional. A-¶139.

2. The Patents And Contemporaneous Literature Establish The Conventionality Of The Claimed Techniques

The Patents repeatedly admit the conventionality of the recited methods of “obtaining a sample” of cfDNA, “genotyping,” “sequencing,” and “detecting” / “quantifying” cfDNA.¹⁹ As discussed above, the Patents even state that “[t]he

¹⁹ The Court previously recognized that the “language in the written descriptions of the . . . asserted patents suggests that the patented steps are neither new nor

practice of the present invention employs, unless otherwise indicated, conventional techniques ... which are within the skill of the art.” UF-10; B0012 at 5:36-48. The Patents also state that each claimed step is “conventional” without “otherwise indicat[ing]” that any step is beyond the ordinary skill. UF-10; A-¶4. Indeed, there is no evidence in the record the Patents claim any improvements to or non-conventional uses of any of the claimed techniques. A-¶69.

In fact, by November 6, 2009, the claimed techniques were a standard way of detecting DNA sequences that, like the donor-specific cfDNA in a transplant patient, differ from a patient’s normal genotype. UF-10; A-¶68. The Patents recount numerous “applications of circulating nucleic acids, [in which] the presence of sequences differing from a patient’s normal genotype has been used to detect disease.” B0012-B0013 at 6:67-7:29; 7:30-32; 7:40-46; 8:18-21; A-¶137. The Patents explain polymorphisms (including SNPs) were routinely used for distinguishing between and detecting one genome from another, noting “any donor and recipient will vary at roughly three million SNP positions if fully genotyped.” B0016 at 13:42-44; B0017 at 16:3-7; A-¶¶93-96. These genetic variations are readily assessed from a biological sample containing cfDNA, and the Patents do not recite any new or improved laboratory techniques for observing them. UF-11-32; A-¶¶129-35. Each claimed step recites techniques that were common practice unconventional[.]” *CareDx, Inc. v. Eurofins Viracor, Inc., et al.*, Case No. 1:19-cv-01804-CFC-CJB, D.I. 53 at 2 (D. Del. Apr. 21, 2020).

by November 2009, and the techniques were performed in a standard order. UF-11-32; A-¶¶129-35.

i. Obtaining a Sample

The Patents recite no improvements for “*obtaining*” or “*providing*” a sample containing cfDNA. UF-12, 30-31; A-¶¶73-77. Rather, the Patents state “[t]o obtain a blood sample, any technique known in the art may be used...” UF-12; B0014 at 10:11-12; B0010 at 1:14-17; B0012 at 6:57-67; B0014 at 9:4-14; B0014 at 10:7-10; A-¶75. CareDx has never argued otherwise, and there is no evidence showing the Claims recite any non-conventional way of obtaining or providing a sample. UF-12, 30-31; A-¶77.

ii. Genotyping / Polymorphism Profile

The Patents—along with contemporaneous literature—establish the claimed “*genotyping*” also was well-known and conventional by November 2009. UF-13, 16; A-¶¶78-81. The Patents state “[g]enotyping of the transplant donor and/or the transplant recipient may be performed by any suitable method known in the art including those described herein such as sequencing ... or PCR.” B0019 at 20:31-37; B0016 at 13:51-67; B0019 at 20:31-51; B0022 at 26:38-41; A-¶80. The particular methodology of genotyping in ’607 element 1(c), “*selective amplification*” to “*amplif[y] ... at least 1,000 [homozygous and heterozygous SNPs] ... by PCR,*” also is described in the Patents as a routine technique scientists

performed in 2009 using off-the-shelf equipment:

Usable SNPs may comprise approximately 500,000 heterozygous donor SNPs and approximately 160,000 homozygous donor SNPs. Companies ... currently offer both standard and custom-designed TaqMan probe sets for SNP genotyping that can in principle target any desired SNP position for a PCR-based assay With such a large pool of potential SNPs to choose from, a usable subset of existing or custom probes can be selected to serve as the probe set for any donor/recipient pair.

B0016 at 13:55-67; A-¶82.

The Patents likewise describe obtaining and using the “*polymorphism*” or “*SNP*” “*profile*” of ’652 element 1(b) and ’497 elements 1(a) and 1(b), or “*using markers distinguishable between [recipient and donor] ... compris[ing] [SNPs]*” of ’607 element 1(f), as standard, stating “after genotyping a transplant donor and transplant recipient, using existing genotyping platforms know [sic] in the art including the ones described herein, one could identify approximately 1.2 million total variations between a transplant donor and transplant recipient.” B0016 at 13:51-55; B0013 at 7:30-36 (“In all these applications of circulating nucleic acids, the presence of sequences differing from a patient’s normal genotype has been used to detect disease.”); A-¶82.

Consistent with the Patents’ disclosures, the contemporaneous literature is replete with examples of commercial products for carrying out the claimed “genotyping,” including for profiling up to thousands of homozygous and heterozygous SNPs, as of the Patents’ November 2009 filing date. UF-32; A-¶¶81,

83-86. The Patents' claims recite no improvements over or non-conventional ways of performing these established approaches to genotyping and polymorphism/SNP profiling. UF-13, 16, 33; A-¶¶78-87.

iii. High-Throughput / Multiplex Sequencing

The record also shows that the claimed sequencing techniques were routine and conventional by November 2009. UF-18-22, 34, 42. The Patents list numerous established commercial providers for “*multiplex*” and “*high-throughput sequencing*” (B0017-B0018 at 15:22-67; 16:58-17:3) and incorporate literature describing the standard use of these techniques. B0017-18 at 15:53-17:14; *see also* B0013 at 7:23-28; B0014 at 9:8-14; B0016 at 14:58-67; B0017-18 at 15:22-17:14; B0020 at 21:5-8; UF-18, 22, 34; A-¶¶100-122. Contemporaneous literature also confirms the conventionality of using these sequencing methods as claimed, including for detecting and quantifying cfDNA. UF-34, 42; A-¶¶100-122. Nothing in the Claims modifies or improves those conventional multiplex or high-throughput sequencing techniques. UF-23, 34, 42; A-¶¶123.

iv. Quantification

The Patents' claimed steps of detecting and quantifying cfDNA were conventional as well. The Patents state that “[d]etection, identification, and/or quantification of the donor-specific markers (e.g., polymorphic markers such as SNPs) can be performed using [numerous techniques]... as well as other methods

known in the art including the methods described herein.” B0014 at 9:8-14; B0018 at 17:41-18:53; B0018-19 at 18:56-19:2 (“Methods for quantifying nucleic acids are known in the art...”); B0020 at 21:5-9; A-¶¶125. These existing, conventional methods also are described in the contemporaneous literature reporting quantification of cfDNA, including quantification of SNPs to detect different genotypes (*e.g.*, in DNA from a fetus, tumor, or infectious disease) in a patient’s sample. UF-16, 22; A-¶¶126-28. Again, the Claims do not modify or claim any improvement over those techniques. UF-16, 22; A-¶¶128.

v. Sensitivity And Error Rate Limitations In The Claims Are Inherent To The Conventional Tools Described In The Patents

Each Claim recites limitations describing test-sensitivity, detection thresholds, and sequencing error rates, but none of these limitations renders any of the Claims patent eligible. Every one of these limitations is inherent to the technologies described in the Patents as routine, conventional, and commercially available. UF-25-29, 39; A-¶¶114-21.

First, the sensitivity limitation in claim 1 of the ’652 patent, which recites the “*sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for ... CAV*,” is conventional. UF-25; A-¶¶114-21. Assuming for purposes of this motion this limitation is not indefinite, it would refer to a degree of sensitivity inherent in the conventional methods of the claims.

Id. It was well-known by 2009 that the number of molecules analyzed is one component of sensitivity, and that higher sensitivity could be achieved by sequencing more molecules. UF-26-27. The Patents, in fact, disclose standard ways to achieve higher sensitivities using the commercially available sequencing equipment without any claimed modification or improvement of that equipment. UF-29; B0018 at 17:12-15; 17:22-25; A-¶¶114-22. The Patents do not disclose or claim any non-conventional uses of this known sequencing equipment. UF-25-29, 35-39.

Second, elements 1(d) of the '497 patent and 1(f) of the '607 patent—each reciting a minimum detection threshold for donor-specific cfDNA—are conventional for similar reasons.²⁰ UF-38; A-¶114. Each Claim recites detection wherein donor-specific cfDNA is “***at least 0.03% of the total circulating [cfDNA] in the biological sample.***” UF-37; A-¶114. The threshold itself describes yet another natural phenomenon—a naturally occurring percentage of cfDNA. A-¶114. The ability to detect that percentage is an inherent property of the commercially available sequencing equipment described in the Patents, a fact the Patents acknowledge when noting that this “[h]igher sensitivity can be achieved ***simply by sequencing more molecules.***” B0018 at 17:1-3; 17:7-13 (also disclosing that the commercially available equipment can detect “donor molecules when the donor

²⁰ '497 patent claims 19-23, and 31-33 and '607 patent claims 7-10 recite similar cfDNA concentration limitations. A-¶¶144, 179, 195-96.

fraction is as low as 0.03%”); A-¶118. And as discussed above, it was well-known by 2009 that by sequencing more molecules, sensitivity will necessarily increase. UF-37-39. The Patents do not disclose or claim any non-conventional laboratory techniques for performing this element.²¹ UF-37-39.

Finally, the '607 patent's recitation of a “**sequencing error rate of less than 1.5%**” is likewise just an inherent property of the commercial sequencers readily available in 2009. B0017 at 16:20-21; A-¶120. In the context of the Patents, the sequencing error rate is an estimate of the proportion of all bases (often expressed as a decimal) sequenced by a sequencer that are incorrect. A-¶¶120-21. These errors can be caused by a combination of problems, for example, during sample preparation, misidentification of single base additions by the sequencer, background noise, or some combination of these and other factors. *See* A-¶¶119-20, 183. As of 2009, it was well-known that typical sequencing error rates for base substitutions varied between sequencing platforms at a rate of 0.5%-1.5%. UF-35. It also was well-known that it was possible to lower the sequencing error rate by resequencing a sample multiple times. UF-36. The Claims recite no improvement

²¹ *See Intellectual Ventures I LLC v. Capital One Fin. Corp.*, 850 F.3d 1332, 1342 (Fed. Cir. 2017) (explaining that “[o]ur law demands more” than claim language that “provides only a result-oriented solution, with insufficient detail for **how** a computer accomplishes it”) (emphasis added); *see also Two-way Media Ltd.*, 874 F.3d at 1337 (finding claims patent ineligible that did “not sufficiently describe **how** to achieve these results in a nonabstract way”); *Credit Acceptance Corp. v. Westlake Servs.*, 859 F.3d 1044, 1057 (Fed. Cir. 2017) (same).

or modification of the commercially available sequencers, and the record does not show that the Patentees developed (let alone claimed) any improvement or unique modification for reducing error rates. A-¶¶114-21.

3. The Prosecution History Establishes The Conventionality Of The Claimed Techniques

The prosecution history further confirms that all of the claimed steps were conventional. A-¶98. During prosecution of the '652 patent, the applicants submitted a declaration stating the steps can be performed using “commercially available kits” and “standard protocols”:

- “DNA from the samples was purified *using commercially available kits*.”
- “Sequencing libraries were constructed from cell-free DNA *using commercially available kits* and sequenced *following standard protocols* using an Illumina GAII sequencer.”
- “Genotypes of the transplant recipients were obtained from genomic DNA using the Illumina Omni1-Quad Beadchip *following standard protocols*.”

B0260-61; A-¶98.²² The Patentees did not argue that obtaining a sample, genotyping, sequencing, or detecting/quantifying were non-conventional or new

²² The PTO recently rejected a pending patent application related to the Patents that is directed to detecting the presence of donor-specific cfDNA on the grounds that claimed detection steps were directed to an abstract “calculative analysis of data” and that claimed diagnosing steps were directed to an abstract “mental step that is the correlation of data and information.” B1314-16 (2019-02-13 Final Rejection, Application No. 14/658,061) at 4-5. The Examiner also determined that recited steps including “steps of providing cell-free DNA and sequencing SNPs” were directed to conventional, well-known elements. B1315.

CareDx’s counsel also confirmed at the Motion to Dismiss hearing the Patents did not “introduce a whole new concept that was never known before to science”:

MR. REINES [CareDX Counsel]: I'm tempted to say that if you isolate down each step or aspect of a step—not each step at the element level, but if you take it *word by word that there's no new concept, that we have not introduced a whole new concept that never was known before to science in any way on a per word basis, I'm tempted to say, yes.*

5. Contemporaneous And Prior Literature Establishes The Conventionality Of The Claims As A Whole

Corroborating the Patentees' admissions,²⁴ prior and contemporaneous literature further confirms that other scientists were applying the claimed techniques to detect DNA sequences that differ from a patient's normal genotype

²³ During the prosecution of the ‘607 patent, the Patentees traversed a Section 101 rejection, but that bears no weight here because (i) the prior art references cited by and relied on by the Examiner are different from the references relied on here, and (ii) the Examiner applied the wrong legal standard—obviousness under 35 U.S.C. § 103—to evaluate those references. *See* B1330.

²⁴ See *supra* Section V(B)(1).

²⁵ B1274 (Lo U.S. 2009) at ¶ [0289]; A-¶152.

2009) at ¶ [0003]; A-¶152. And the prior art method disclosed the same conventional steps claimed here. B1251, B1254, B1258, B1263, B1271-74 (Lo U.S. 2009) at ¶¶ [0003], [0059-60], [0100], [0176], [0262], [0278]; [0289]; A-¶153.

There are numerous other examples showing the Claims' techniques together as a whole were known and conventional by 2009 that are further discussed in the Quackenbush declaration. *See* B0428-B0435 (Dhallan 2007); B0436-B0444 (Beck 2009); B0454-B0460 (Gordon 2009); B0461-B0462 (Holt 2009); A-¶¶154-68. Accordingly, there is nothing non-conventional about the Claims' recitation, as a whole, of well-known scientific techniques,²⁶ and there is no evidence to the contrary. *See, e.g., Ass'n for Molecular Pathology v. U.S. Patent & Trademark Office*, 689 F.3d 1303, 1334 (Fed. Cir. 2012); A-¶¶154-68.

6. The Dependent Claims Do Not Recite Inventive Concepts

None of the dependent limitations are described in the written description as non-conventional. To the contrary, according to the specification and as confirmed by Natera's expert Dr. Quackenbush, the dependent limitations all either add information about the natural phenomenon or recite conventional laboratory techniques. A-¶¶169-99.

²⁶ *Bascom Glob. Internet Servs., Inc. v. AT&T Mobility LLC*, 827 F.3d 1341, 1348-49 (Fed. Cir. 2016).

- *Dependent Claims Reciting Different Polymorphisms* ('652 patent at claims 2, 11; '497 patent at claims 6, 17, 25; '607 patent at claims 2, 3, 4, 5). As explained above, genotyping to obtain a polymorphism profile comprising SNPs of various quantities was well-understood, routine and conventional as of November 2009. UF-13-22; A-¶¶78-79, 170-73.
- *Dependent Claims Reciting Types Of cfDNA and cfRNA* ('652 patent at claim 3; '497 patent at claim 12). As the Patents admit, it was routine and conventional as of November 2009 to assay any of the recited types of natural occurring nucleic acids using the claimed methods. UF-3-6, 16-17; A-¶¶174-77.
- *Dependent Claims Reciting Certain Concentrations Of Cell-Free Nucleic Acids In The Sample* (See '497 patent at claim 20; '607 patent at claims 7, 8). As explained above, detecting the claimed concentrations of cell-free nucleic acids was an inherent feature of the standard and conventional methods and instruments disclosed by the Patents. See *supra* Section V(B)(2); UF-12, 34, 37-39; A-¶¶195-97.
- *Dependent Claims Reciting Certain Common Modifications, Error Rates, Or Quality Scores Associated With Multiplexed Or High Throughput Sequencing* ('652 patent at claim 6; '497 patent at claims 3, 4, 5, 19, 21, 23; '607 patent at claim 6). It is evident from the Patents, literature, and equipment manuals that all of these limitations were either conventional methods or features inherent to the techniques of the independent claims. UF-34-32; A-¶¶178-85 (discussing each limitation).
- *Dependent Claims Reciting Further Genotyping Prior To Or Simultaneously With Quantifying Cell-Free Nucleic Acids* ('497 patent at claims 15). The written description and Claims do not disclose or claim any non-conventional application of these genotyping limitations. A-¶¶194. Nor do they disclose or claim improvements to conventional genotyping techniques as of 2009 that make this claim inventive. *Id.*
- *Dependent Claims Reciting Organ or Species Limitations* (See '652 patent at claims 14, 15; '497 patent at claim 9). The Patents do not disclose or claim anything non-conventional or inventive about selecting a particular type of transplant organ (*e.g.*, kidney, pancreas, heart, etc.). UF-10, 12, 17; A-¶¶186-88. Nor do the Patents state or claim they

invented analyzing the bodily fluid samples of human transplant recipients as opposed to those of some other species. *Id.*

- *Dependent Claims Reciting Conventional PCR Or Amplification Methods* (See '497 patent at claim 10). As explained above, digital PCR, real-time PCR, and use of arrays were well-understood, routine and conventional as of November 2009. UF-13, 15-24; A-¶¶189-90. And performing an amplification reaction on donor cfDNA prior to quantifying it also was routine and conventional. *Id.*
- *Dependent Claims Reciting Different Sample Types* ('652 patent at claims 12; '497 patent at claims 2, 27). Using patient samples from naturally occurring sources such as blood, serum, urine, stool, or plasma to extract cfDNA does not confer patentability either. UF-3-5; 11-23; A-¶¶191-93. Obtaining a biological sample from a patient—from whatever natural source—and using it with the claimed methods was standard and routine by 2009. *Id.*

C. The Claims Do Not Become Patentable By Limiting The Field Of Use

By CareDx's admission, the only purported innovation in the Claims is the application of the claimed method *to the particular field of transplant cfDNA*.²⁷ But that is not enough to confer patentability. "As the Supreme Court has held, 'the prohibition against patenting abstract ideas [or natural phenomena] cannot be circumvented by attempting to limit the use of the formula to a particular technological environment.'" *Molecular Pathology*, 689 F.3d at 1334 (citing *Bilski v. Kappos*, 561 U.S. 593, 610 (2010)) (finding that "[l]imiting [a] comparison to just the *BRCA* genes or ... the identification of particular alterations," *i.e.*,

²⁷ D.I. 17 (Furneaux Decl.) at ¶ 25 ("It was not well-known or conventional to use these techniques *together*, in 2009, *in the context of non-invasive diagnostic tests for organ transplant rejection*.").

particular manifestations of natural phenomena regarding genetic mutations, “fails to render the claimed process patent-eligible.”). Accordingly, CareDx’s field of use argument cannot render the Claims patentable.

Numerous other courts have found that limiting the field of use of conventional techniques to specific categories of natural phenomenon—like the category of donor-specific cfDNA here—is insufficient to confer patentability.²⁸ *Genetic Techs.* and *Ariosa* are particularly analogous. In both cases, the Federal Circuit held that various “physical steps” such as the “physical steps of DNA amplification and analysis of the amplified DNA” and “PCR to amplify and detect [the cell-free DNA]” were all well-understood and conventional—much like the physical steps of PCR/amplification, sequencing, and genotyping here. *See Genetic Techs.*, 818 F.3d at 1377-78; *Ariosa*, 788 F.3d at 1377. This is why, in *Ariosa*, the court evaluated whether it was “well-understood, routine, and conventional activity” to combine the recited method steps to detect DNA in blood *generally*, not whether it was routine to apply those steps to a particular kind of DNA—*maternal and fetal cfDNA*—which was the natural phenomenon at issue there.

²⁸ *See, e.g., Alice*, 573 U.S. at 222-23; *Mayo*, 566 U.S. at 84; *Bilski*, 561 U.S. at 612; *Affinity Labs of Texas, LLC v. DIRECTV, LLC*, 838 F.3d 1253, 1259 (Fed. Cir. 2016); *Content Extraction*, 776 F.3d at 1348; *buySAFE, Inc. v. Google, Inc.*, 765 F.3d 1350, 1355 (Fed. Cir. 2014); *Exergen Corp. v. Thermomedics, Inc.*, 132 F. Supp. 3d 200, 207 (D. Mass. 2015).

Ariosa, 788 F.3d at 1377.²⁹

The same result is warranted here. The Claims are directed to a particular natural phenomenon—donor-specific cfDNA that circulates in the blood of a transplant recipient—and recite standard laboratory techniques to detect that natural phenomenon. A-¶¶59-199. But, as in *In re BRCA*, “[n]othing is added by identifying the techniques to be used . . . because those . . . techniques were the well-understood, routine, and conventional techniques that a scientist would have thought of when instructed” to detect and analyze cfDNA in a transplant recipient’s body. 774 F.3d at 764; A-¶¶66.

D. The Claims Are Analogous To Diagnostic Claims The Supreme Court And Federal Circuit Have Invalidated

1. Analogous Cases Confirm Unpatentability Of The Claims

As this Court has recognized, “[t]o determine whether claims are directed to an abstract idea courts generally ‘compare the claims at issue to those claims already found to be directed to an abstract idea in previous cases.’” *In-Depth Test, LLC v. Maxim Integrated, Prods., Inc.*, C.A. No. 14-887-CFC, 2018 WL 6617142, at *4 (D. Del. Dec. 18, 2018) (quoting *Enfish, LLC v. Microsoft Corp.*, 822 F.3d 1327, 1334 (Fed. Cir. 2016)). Here, comparing the Claims to those

²⁹ See also *In re BRCA*, 774 F.3d at 764 (finding unpatentable claims that recited “detecting,” “amplifying,” and “sequencing” genes because those techniques did “nothing more than spell out what practitioners already knew—how to compare gene sequences using routine, ordinary techniques”).

previously found unpatentable by the Supreme Court and Federal Circuit further confirms the Claims are directed to a natural phenomenon and add no inventive concept. *See Illumina*, 952 F.3d at 1371 (“[W]e have consistently held diagnostic claims unpatentable as directed to ineligible subject matter.”).

In *Mayo*, the Supreme Court invalidated claims “set[ting] forth laws of nature—namely, relationships between concentrations of certain metabolites in the blood and the likelihood that a dosage of a [] drug will prove ineffective or cause harm.” 566 U.S. at 77. Here too, the Claims set forth laws of nature—namely relationships between the amounts of donor-specific cfDNA detected in the blood and, in the case of the ‘652 patent, the likelihood that a transplanted organ is being rejected or failing. A-¶¶59-66.

In *Athena*, the Federal Circuit found methods for detecting auto-antibodies produced in patients suffering from a certain autoimmune disease to be “directed to” a natural law, holding “[c]laiming a natural cause of an ailment and well-known means of observing it is not eligible for a patent because such a claim in effect only encompasses the natural law itself.” 915 F.3d at 752-53. Similarly here, the Patents claim a natural correlation to an ailment—increased levels of transplant donor-specific cfDNA circulating within a transplant recipient—and only well-known means of observing it. A-¶¶59-199.

Similarly in *Cleveland Clinic*, the Federal Circuit found that claims

detecting “elevated levels” of a natural inflammatory enzyme called “MPO,” and the correlation of this MPO to the likelihood of cardiovascular disease—were “directed to” a natural phenomenon. 859 F.3d at 1360-61. The court found they were not directed to a patentable laboratory method for detecting MPO because “[t]he specifications of the testing patents confirm that known testing methods could be used to detect [the natural phenomenon], and that there were commercially available testing kits for [the natural phenomenon] detection.” *Id.* at 1361. The same is true here, where the Claims recite detecting elevated levels of donor-derived cfDNA in a transplant recipient’s blood, but again recite no improvements over the known—and commercially available—methods recited in the Claims. A-¶¶73-168.

In *Ariosa Diagnostics*, the Federal Circuit found claims for detecting naturally occurring fetal cfDNA in a pregnant woman’s blood using “method steps [that] were well-understood, conventional and routine” to be directed to a natural phenomenon. 788 F.3d at 1376-77. Specifically, the claims recited steps such as “PCR to amplify and detect cfDNA” that the court found conventional as of 1997. *Id.* at 1377. Here, as well, the Claims recite detecting naturally occurring quantities of donor-specific cfDNA in a transplant recipient’s blood without reciting any improvement over the well-understood, conventional, and routine detection methods recited in the claims. A-¶¶73-127.

And in *Genetic Techs.*, the Federal Circuit invalidated a claim for detecting compilations of genetic sequences by taking advantage of how certain sequences were naturally “linked” to others. 818 F.3d at 1374-76. The Court found that claim to be “directed to” a natural law because it “does not purport to identify novel detection techniques.” *Id.* at 1376. Similarly here, the Patents claim methods for detecting selected compilations, or profiles, of genetic sequences (polymorphisms or SNPs) in cfDNA of a transplant recipient without identifying any new or improved techniques for the detection. A-¶¶67-199; *see also Genetic Veterinary Scis., Inc. v. LABOKLIN GmbH & Co. KG*, 933 F.3d 1302, 1315-18 (Fed. Cir. 2019) (claim reciting “method for genotyping” with steps of “obtaining” a sample, “genotyping” a gene sequence and “detecting” specific nucleotide replacement were directed to natural law).

2. *CellzDirect* And *Illumina* Are Not Applicable

In two recent life sciences cases, the Federal Circuit upheld the patentability of certain non-analogous claims. *See Rapid Litig. Mgmt. Ltd. v. CellzDirect*, 827 F.3d 1042 (Fed Cir. 2016); *Illumina, Inc. v. Ariosa Diagnostics, Inc.*, 952 F.3d 1367 (Fed. Cir. 2020). Those cases do not apply here for at least two reasons.

a. Unlike *CellzDirect* and *Illumina*, The Claims Here Do Not Lead To An Unnatural Result Or Preparation

First, the claims upheld in *CellzDirect* and *Illumina* claimed the use of a

natural phenomenon to arrive at an unnatural result, which is not true here. In *Illumina*, the claims were directed to “methods for preparing **a fraction** of cell-free DNA **that is enriched in fetal DNA**” by taking advantage of the fact that fetal cfDNA tends to be smaller than maternal cfDNA. *Illumina*, 952 F.3d at 1372. The *Illumina* claims recited “specific process steps” for performing DNA size discrimination and selectively removing DNA fragments that were above a specific size threshold, which thereby increased the relative amount of fetal DNA as compared to maternal DNA in the sample. *Id.* The claimed process “**change[d] the composition of the mixture**, resulting in a DNA fraction that is **different from the naturally-occurring fraction** in the mother’s blood.” *Id.*

In *CellzDirect*, the claimed methods took advantage of the ability of certain liver cells to survive multiple cycles of freezing and thawing. 827 F.3d at 1045-46. The claimed methods took advantage of this natural survivability and claimed a process for preserving liver cells by preparing a select and unnatural population of liver cells resilient to cryopreservation. *Id.* at 1050-52. Specifically, the improved process involved freezing cells, thawing cells, removing non-viable cells, and then refreezing the viable cell. *Id.* The result of this process created an unnatural composition of cells not found in nature. *Id.* at 1048-51.

In contrast to *Illumina* and *CellzDirect*, the Claims here do not result in any unnatural “preparation” or “composition.” Unlike *Illumina* and *CellzDirect*, the

Claims do not selectively remove, alter, or modify cfDNA to change the composition of the samples into something else. Altering the samples in the ways claimed by *Illumina* and *CellzDirect* would, in fact, stifle the purpose of the Claims, which is to detect naturally occurring levels of donor and recipient cfDNA in a transplant patient and compare naturally occurring differences between them to assess transplant status. A-¶¶59-66. The Claims thus do not produce something “different from the naturally-occurring [cfDNA] fraction,” but instead do the opposite—they observe a naturally occurring cfDNA fraction. *Illumina*, 952 F.3d at 1372.

b. Unlike CellzDirect and Illumina, The Claims Here Do Not Disclose Any New Or Improved Laboratory Technique

Second, in *CellzDirect* and *Illumina*, the Federal Circuit held the claims recited new and improved laboratory techniques, which the Claims here do not recite. A-¶¶69-199. Notably, the Federal Circuit itself in *Athena* and *Cleveland Clinic* distinguished *CellzDirect* on grounds that apply equally here—the *CellzDirect* claims were targeted to a “new and useful laboratory technique.” See *Cleveland Clinic*, 859 F.3d at 1361 (distinguishing *CellzDirect*); *Athena*, 915 F.3d at 751-52 (same); see also Section V(B) above (demonstrating standard use of Patents’ recited laboratory methods). In *CellzDirect*, the improvement involved freezing and thawing viable liver cells more than once (as well as pooling cells)—

techniques the contemporaneous literature discouraged. *CellzDirect*, 827 F.3d at 1048-49, 1051 (“Repeating a step that the art taught should be performed only once can hardly be considered routine or conventional”). In *Illumina*, the improvement involved setting size thresholds to segregate the cfDNA such that fetal cfDNA was enriched in the final preparation. *Illumina*, 952 F.3d at 1375. But here, the Patents recite no improvements or non-conventional techniques—only the routine and conventional techniques of obtaining a sample, genotyping, sequencing cfDNA, and determining/quantifying donor-specific cfDNA. That is confirmed by the Patentees own admission that “[t]he practice of the present invention employs, unless otherwise indicated, **conventional techniques** ... which are within the skill of the art.” B0012 at 5:36-49.

VI. CONCLUSION

For the foregoing reasons, Natera requests the Court find the Claims of the Patents unpatentable under 35 U.S.C. § 101 and dismiss the FAC in its entirety with prejudice.

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CERTIFICATE OF COMPLIANCE

I hereby certify that this brief has been prepared in Times New Roman 14-point typeface using Microsoft Word, and contains 9,668 words as determined by the Word Count feature of Microsoft Word.

June 11, 2020

/s/ Derek J. Fahnestock

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CERTIFICATE OF SERVICE

I hereby certify that on June 11, 2020, I caused the foregoing to be electronically filed with the Clerk of the Court using CM/ECF, which will send notification of such filing to all registered participants.

I further certify that I caused copies of the foregoing document to be served on June 11, 2020, upon the following in the manner indicated:

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Exhibit 12

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

BEFORE THE PATENT TRIAL AND APPEAL BOARD

Natera Inc.

Petitioner

v.

Illumina, Inc.

(record) Patent Owner

Case No. IPR2018-01317

U.S. Patent No. 9,493,831

DECLARATION OF MICHAEL L. METZKER, Ph.D.

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I, Michael L. Metzker, Ph.D., declare as follows:

I. INTRODUCTION

1. I, Michael L. Metzker, have been retained as an expert witness on behalf of Natera, Inc. (the “Petitioner”) in connection with the above-captioned *Inter Partes* review (“IPR”). I understand that the petition for IPR involves U.S. Patent No. 9,493,831 (the “’831 Patent,” (Ex. 1001)), which resulted from U.S. Application Ser. No. 14/677,854, (the “’854 Application”) filed on April 2, 2015, on behalf of Yue-Jen Chuu and Richard P. Rava.

II. MATERIALS REVIEWED

2. I have reviewed the materials cited herein, including the following exhibits. The materials are publications that were typically read and reasonably relied upon by persons of ordinary skill in this field, including the bibliographic information presented therein.

Exhibit No.	Description
1001	U.S. Patent No. 9,493,831 (“ the ’831 Patent ”).
1002	Declaration of Dr. Michael Metzker, Ph.D.
1003	U.S. Pat. App. Pub. US 2010/0120038 A1 (“ Fluidigm ”).
1004	U.S. Pat. App. Ser. No. 12/548,132 (“ Fluidigm App ”).
1005	WO 2009/032781 A2 (“ Sequenom ”).
1006	Fan, et al., <i>Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood</i> , Proc. Natl. Acad. Sci. USA, Vol. 105:16266–16271 (October 21, 2008)(“ Fan 2008 ”).

1007	Harismendy, et al., <i>Evaluation of next generation sequencing platforms for population targeted sequencing studies</i> , Genome Biology, Vol. 10:R32 (March 27, 2009)(“ Harismendy ”).
1008	Bentley, et al., <i>Accurate whole human genome sequencing using reversible terminator chemistry</i> , Nature, Vol. 456:53-59 (November 2008)(“ Bentley ”).
1009	Curriculum vitae of Dr. Michael Metzker, Ph.D.
1010	File History of U.S. Pat. App. Ser. No. 14/677,854.
1011	U.S. Pat. No. 8,318,430.
1012	Quail, et al., <i>A large genome center’s improvements to the Illumina sequencing system</i> , Nature Methods, Vol. 5:1005-1010 (Dec. 2008)(“ Quail ”).
1013	Chiu, et al., <i>Noninvasive Prenatal Diagnosis of Fetal Chromosomal Aneuploidy by Massively Parallel Genomic Sequencing of DNA in Maternal Plasma</i> , Proc. Natl. Acad. Sci. USA, Vol. 105:20458-20563 (Dec. 23, 2008)(“ Chiu ”).
1014	Illumina, Inc., <i>Multiplexed Sequencing with the Illumina Genome Analyzer System</i> ”, Illumina, 2008 (“ Illumina Datasheet ”).
1015	Declaration of Sylvia Hall-Ellis.
1016	Illumina, Inc., <i>Preparing Samples for Multiplexed Paired-End Sequencing</i> (December 2008)(“ Illumina Guide ”).
1017	IDS filing of March 11, 2013 in U.S. App. Ser. No. 13/792,661.
1018	Saiki et al., <i>Enzymatic amplification of β-globin genomic sequences and restriction site analysis for diagnosis of sickle cell anemia</i> , Science, Vol. 230:1350-1354 (Dec. 20, 1985)(“ Saiki 1985 ”).
1019	Saiki et al., <i>Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase</i> , Science, Vol. 239:487-491 (Jan. 29, 1988)(“ Saiki 1988 ”).

1020	Gibbs et al., <i>Detection of single DNA base differences by competitive oligonucleotide priming</i> , Nucleic Acids Research, Vol. 17:2437-2448 (Apr. 11, 1989)(“ Gibbs 1989A ”).
1021	Newton et al., <i>Analysis of any point mutation in DNA. The amplification refractory mutation system (ARMS)</i> , Nucleic Acids Research, Vol. 17:2503-2516 (1989)(“ Newton ”).
1022	Chamberlain et al., <i>Deletion screening of the Duchenne muscular dystrophy locus via multiplex DNA amplification</i> , Nucleic Acids Research, Vol. 16:11141-11156 (1988)(“ Chamberlain ”).
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1046	Huber et al., <i>Microbial population structures in the deep marine biosphere</i> , Science, Vol. 318:97-100 (2007)(“ Huber ”).
1047	McKenna et al., <i>The macaque gut microbiome in health, lentiviral infection, and chronic enterocolitis</i> , PLOS Pathogens, Vol. 4:e20 (2008)(“ McKenna ”).
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1054	Tewhey et al., <i>Microdroplet-based PCR enrichment for large-scale targeted sequencing</i> , Nature Biotechnology, Vol. 27:1025-1031 (2009)(“ Tewhey ”).
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III. SUMMARY OF OPINIONS

3. In my opinion, claims 1-10, 12-22 and 24 of the '831 Patent were unpatentable as obvious. My opinion is based on the following four grounds:

- a. Ground 1: Claims 1-7, 10, 14-19 and 22 were obvious over Fluidigm in view of the knowledge of one of ordinary skill;
- b. Ground 2: Claims 1-10, 13-22, and 24 were obvious over as in Ground 1 taken together with Sequenom

c. Ground 3: Claim 12 was obvious as in Ground 1, together with Harismendy.

d. Ground 4: Claim 12 was obvious as in Ground 2, together with Harismendy.

IV. EXPERIENCE AND QUALIFICATIONS

4. I am currently Founder, President, and Chief Executive Officer of RedVault Biosciences, LP (“RedVault”). RedVault is a new biotechnology company committed to creating innovative technologies to advance genomic medicine, which I founded in April 2013. Since starting RedVault, I have transitioned into an Adjunct Associate Professor in the Department of Molecular & Human Genetics and at the Human Genome Sequencing Center (“HGSC”) at the Baylor College of Medicine. Additionally, I am an Adjunct Associate Professor in the Department of Chemistry at Rice University.

5. Prior to my current positions, I was a tenured Associate Professor in the Department of Molecular & Human Genetics and at the HGSC at the Baylor College of Medicine. I have also been a Senior Manager at the HGSC at Baylor College of Medicine. I have also been an Adjunct Associate Professor in the Cell & Molecular Biology Program at the Baylor College of Medicine. Prior to my tenured positions, I held the position of tenure-track Assistant Professor in Molecular and Human Genetics and Senior Manager at the HGSC since November 1999 and the position

of Adjunct Assistant Professor in Chemistry at Rice University since July 2001.

6. In March 2002, I founded LaserGen, Inc. (“LaserGen”), which provides technology and reagents used in, among other things, nucleic acid sequencing methods. From March 2002 to April 2012, I was President and Chief Executive Officer of LaserGen, and from April 2012 to October 2012, I held the position of Chief Technology Officer. I was also a member of the Board of Directors from March 2002 to October 2012. I currently have no working relationship with LaserGen. In March 2016, Agilent Technologies invested \$80M into LaserGen to develop its reversible terminator technology into a clinical diagnostic platform. In March 2018, Agilent Technologies purchased the remaining shares of LaserGen for \$105M.

7. Since 1988, I have performed scientific research in the field of molecular biology. In particular, I have extensive experience with technology development in DNA sequencing methods including genomic DNA isolation, polymerase chain reaction (“PCR”), fragmenting genomic DNA, genomic DNA and complementary DNA (“cDNA”) library construction, bacterial cloning, nucleic acids chemistry, DNA modifying enzymes, polymerase engineering, fluorescent dyes, fluorescence imaging, and data analysis of multi-color images. I have extensive experience in the use and development of methods utilized to detect sequence variation, including single nucleotide variants (“SNVs”) and single

nucleotide polymorphisms (“SNPs”), in all organisms, with particular interest in humans and human immunodeficiency virus (“HIV”).

8. My research has been devoted to developing next-generation sequencing (“NGS”) technologies, developing novel methods to study HIV transmission between individuals, and identifying molecular causes of novel forms of diabetes and their treatment. My colleagues and I have been deeply involved in PCR, DNA fragmentation, library construction, cloning, and the development of sequencing technologies. My industrial experience entails performing analytical chemical testing of inorganic materials (Aerojet-General), organic synthesis and characterization of porous polystyrene beads for applications in high performance liquid chromatography (Bio-Rad), development and applications of a robotic platform in Sanger sequencing (Applied Biosystems), and development and applications of high-throughput DNA sequencing to identify novel gene targets for small molecule screening (Merck Research Laboratories).

9. I received my Bachelor of Science degree in Biochemistry & Biophysics from the University of California, Davis in 1984. Between 1985 and 1988, I completed the required coursework with emphasis in Organic Chemistry as part of the Master of Science program in the department of Chemistry at San Francisco State University. Instead of completing this degree, I pursued a Doctor of Philosophy in 1991 from the Department of Molecular and Human Genetics at

Baylor College of Medicine in Houston, Texas. I received my Ph.D. in 1996 in Molecular and Human Genetics.

10. From 2000 to the present, I have given lectures to graduate and medical students in the Molecular Methods course at the Baylor College of Medicine. The lectures are titled, cDNA and Genomic Libraries, First-generation Sequencing, and Genotyping, and Next-generation Sequencing. From 2001 to 2003, I also gave a lecture titled Mammalian Genome Analysis in the Mammalian Genomics.

11. I have also presented lectures at a variety of academic and industry conferences. In May 2010, I was an invited speaker at the Next-Generation Sequencing Workshop at Lübeck University in Germany. In October 2010, I was an invited speaker at the Centre de Regulació Genòmica (“CRG”) Symposium, Barcelona Spain. In April 2011, I was keynote speaker at the Next-Generation Sequencing Conference in Boston, MA. In February 2012, I gave a presentation related to the development of a novel NGS technology at the Advances in Genome Biology and Technology Meeting (“AGBT”) in Marco Island, FL. I was also invited to speak at the Copenhagenomics Meeting in Copenhagen, Denmark and the American Society of Microbiology Conference in San Francisco, CA both in June of 2012. In May 2015, I presented a keynote lecture at SelectBio’s Advances in Next Generation Sequencing Meeting. In April 2016, I was invited to speak at the Critical Path to TB Drug Regimens (“CPTR”) Workshop in Washington DC.

12. I have reviewed numerous manuscripts for peer-review publications that present data for many of the techniques described above. I have also authored several extensive reviews on sequence technologies and their applications used for the detection of sequence variation, including SNPs and SNVs. I have also received research grants and fellowships both from government and private agencies to fund my investigations of technology development in Sanger sequencing and NGS methods, development of NGS approaches for HIV forensics, the detection of genomic variation in diabetic cohorts using novel methods, and technology development of a novel diagnostic method involving microRNAs. I have received or have been associated with numerous grants largely focusing on gene and genome sequencing totaling over \$170 million since 1999. The source of these grants has been the National Institutes of Health (“NIH”), the National Institute of Justice (“NIJ”), the United States Department of Agriculture (“USDA”), and private foundations.

13. I have authored 56 peer-reviewed papers, seven book chapters, and am an inventor on 49 issued patents and patent applications. My most recent review papers discuss the emerging technologies and advances in DNA sequencing. The patents relate to various aspects of molecular biology, DNA sequencing, and chemical synthesis. For example, I co-invented the pulsed-multiline excitation (“PME”) method in collaboration with Nobel Laureate Robert F. Curl, which has

resulted in U.S. Patent Nos. 6,995,841; 7,511,811; and 8,089,628. By way of collaboration with my scientific team at LaserGen, I have also co-invented novel nucleotide terminators that have resulted in numerous patents including U.S. Patent Nos. 7,893,227; 7,897,737; 7,964,352; 8,148,503; 8,198,029; 8,361,727; 8,497,360; 8,877,905; 8,889,860; 8,969,535; 9,200,319; 9,399,798; 9,689,035.

14. I have served on review panels for Genome Canada, Canadian Institute for Health Research (“CIHR”), National Aeronautics and Space Administration (“NASA”), and U.S. Department of Energy (“DOE”), as well as various NIH study sections for the National Cancer Institute (“NCI”), National Institute of Biomedical Imaging and Bioengineering (“NIBIB”), National Human Genome Research Institute (“NHGRI”), and National Institute for Allergy and Infectious Diseases (“NIAID”). In March 2018, I became a member of the College of Reviewers for CIHR.

15. From 2003 to 2006, I served on the editorial/advisory boards for Genome Research. From 2006 to 2012, I have served as a scientific organizer for the AGBT Meeting. From 2007 to present, I have served as a scientific advisor for Milestones in DNA Technologies. From 2011 to 2013, I have served on the advisory committee of Genome Canada: Advancing Technology Innovation Through Discovery (“ATID”) projects – Finding of Rare Disease Genes in Canada (“FORGE Canada”) and the Canadian Pediatric Cancer Genome Consortium (“CPCGC”).

16. I belong to the American Association for the Advancement of Science, the American Chemical Society, and the Texas Genetics Society. My professional experience, education, and publications are presented in greater detail in my curriculum vitae, including publications and patents, as well as cases that I have provided expert testimony at deposition or trial in the last four years. (Ex. 1009).

17. As an expert in molecular biology, I am qualified to provide an opinion as to what one of ordinary skill in the art would have understood, known or concluded as of the 2010 time frame or several years prior to that.

V. TECHNICAL BACKGROUND

A. Introduction to the '831 Patent

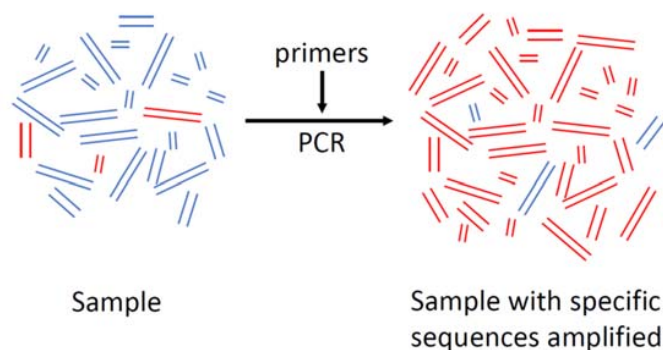
18. The '831 Patent relates to the preparation of sequencing libraries. A sequencing library has a collection of polynucleotide sequences which can be, for example, genomic or cell-free DNA. The polynucleotide sequences are usually taken from some biological sample, such as a blood sample. In many biological samples, there will be a large amount of polynucleotides, including genomic or cell-free DNA. Some of these sequences might be of interest to a medical professional or patient. For example, the presence (or elevated / lowered) amounts of sequences associated with a particular disorder could be used for a diagnosis.

19. To detect sequences of interest from among the (generally) much larger collection of sequences found in a sample in the relevant timeframe (and still today),

a general and multi-step process was used. The first step of the general process was to prepare the sample into a library of sequences. A library preparation method typically has several processing steps that can also include *selectively amplifying* sequences of interest. To selectively amplify sequences of interest, the polymerase chain reaction (PCR) can be used. A more complete introduction to PCR technology is provided in ¶¶23-44, below.

20. PCR uses primer sequences. The primers will bind to sequences in the sample under appropriate conditions, if those sample sequences have reasonably complementary base pairs. Included in a PCR reaction is a DNA polymerase. An illustration of the PCR process in

general is shown at right (created by the Petitioner), in which a sample on the left containing DNA sequences (shown as two

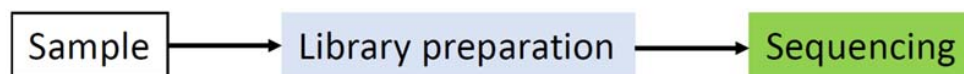


complementary strands) have sequences of interest (shown in red), and sequences that are not of interest (shown in blue). By applying PCR with primer pairs directed to the sequences of interest, a reaction product (right side) is obtained that has the sequences of interest selectively amplified (the size of the samples and number of strands are not meant to be accurate, but only to illustrate the process at a general level). By using multiple rounds of PCR, the sequences of interest can be amplified

to a point where they become dominant sequences in the sample.

21. Using PCR in this fashion, one could amplify target sequence to create a sequencing library. The sequencing library can then be used in a *sequencing analysis* of the amplified target sequences by determining the base-pair order of the polynucleotides in the library. Therefore, the presence, absence and concentration of target sequences in the library can provide information about the original sample, including the disease state(s) of the individual from whom the sample was taken.

22. Two general steps of such a diagnostic process (library preparation and sequencing) are shown below:



23. One example of such a diagnostic process using library preparation and sequencing begins with a sample of maternal blood plasma from a pregnant woman. As was well-known prior to the earliest possible benefit date of the '831 Patent, a pregnant mother's blood contains both her own cell-free DNA and cell-free DNA from the fetus. (Ex. 1006, p. 001)(Ex. 1013, p. 001)(Ex. 1005, 2:29-3:10)(Ex. 1003, ¶0123). One could take, for example, a sample of the maternal plasma and selectively amplify DNA sequences from chromosome 21, thereby allowing one to test for Down syndrome in the fetus. Down syndrome is caused by an extra copy of chromosome 21 (a so-called "trisomy" of chromosome 21). An extra copy of

sequences from chromosome 21 in the blood of a mother without Down syndrome indicates a higher probability Down syndrome in the fetus.

24. The claims of the '831 Patent are directed to the first step of the diagnostic process: library preparation. In general, the claims require preparing a sequencing library using three amplification steps. For example, claim 1 of the '831 Patent is reproduced below, with the three amplifications steps bolded:

“1. A method for preparing a sequencing library from a maternal blood sample, the method comprising:

a. obtaining a maternal blood sample comprising fetal and maternal cell-free DNA;

b. selectively enriching a plurality of non-random polynucleotide sequences of genomic DNA from said fetal and maternal cell-free DNA to generate a library of enriched non-random polynucleotide sequences, wherein said plurality of non-random polynucleotide sequences comprises at least 100 different non-random polynucleotide sequences selected from a chromosome tested for being aneuploid, said enriching comprising:

(i) **a first amplification step** to generate a plurality of first reaction products, said amplification comprising at least 100 first primers configured to amplify at least 100 different non-random polynucleotide sequences;

(ii) **a second amplification step** to generate a second reaction product, said amplification comprising a second set of primers comprising sequences contained in the first reaction products; and

(iii) **a third amplification step** to generate a third reaction

product comprising said library of enriched non-random polynucleotide sequences, said amplification comprising a third set of primers comprising sequences contained in the second reaction products;

wherein at least one primer of at least one of the second and third sets of primers includes a sequence configured to be added to the different non-random polynucleotide sequences to permit the enriched non-random polynucleotide sequences of the library to anneal to a same sequencing primer for the enriched non-random polynucleotide sequences of the library.”

B. DNA Basics

25. The information necessary to reproduce a given organism is found in its genome, which comprises the complete set of an organism’s genes. The genome is comprised of strands of a polymer called deoxyribonucleic acid or “DNA.” Each cell for a given organism typically contains one copy of the genome, called genomic DNA.

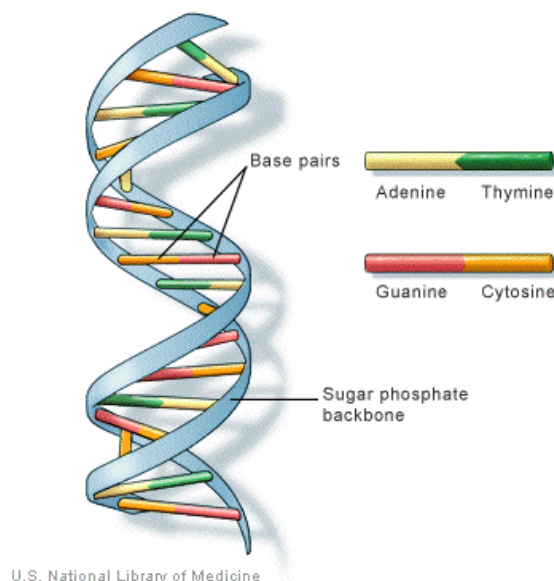
26. DNA strands are polymers of building blocks called nucleotides. A nucleotide is made up of three chemical components or groups: the nucleobase (commonly referred to as a base), the sugar, and one or more phosphate groups. For DNA, the specific sugar group is a 2’-deoxyribose. For ribonucleic acid (“RNA,”) the specific sugar group is a ribose. In addition to this difference, DNA contains the base thymine (“T”) while RNA contains the base uracil (“U”). Using an enzyme

called a polymerase, nucleotides that are added during DNA synthesis are called 2'-deoxyribonucleoside-5'-triphosphates or "dNTPs," and those added during RNA synthesis are called ribonucleotide-5'-triphosphates or "NTPs" or sometimes "rNTPs."

27. A polymerase that synthesizes DNA is called a DNA polymerase and one that synthesizes RNA is called a RNA polymerase. Polymerases can possess other activities in addition to synthesizing nucleic acids. For example, a polymerase can remove nucleotides from one or the other end of nucleic acids. Polymerases that remove nucleotides from the 5'-end of a nucleic acid has a 5'-exonuclease activity. Polymerases that remove nucleotides from the 3'-end of a nucleic acid has a 3'-exonuclease activity.

28. The DNA structure can be characterized as a ladder. The two opposing legs of the DNA ladder are based on a repeated pattern of a sugar group and a phosphate group, called the DNA backbone. The chemical variance occurs in the rungs of the DNA ladder—i.e., the four different bases: adenine ("A"), cytosine ("C"), guanine ("G"), and thymine ("T")—which form hydrogen bonds according to specific base-pairing rules. "A" and "T" can form stable hydrogen bonds, and "C" and "G" can form stable hydrogen bonds. Said in another way, "A" does not pair with "G" and "C" does not pair with "T." For RNA, "U" is substituted for "T" whereby "A" and "U" can form stable hydrogen bonds.

29. The formation of base-pairs between nucleotides on the two opposite strands causes the two strands to coil around each other to form a double helix structure, first described by James Watson and Francis Crick in 1953. Bases of nucleotides that follow these base-pairing rules are also called Watson-Crick base-pairs (“bp”). An example of a double helix is shown below.



30. According to the base-pairing rules, stretches of nucleotides in one strand can form hydrogen bonds only with a complementary stretch of nucleotides in the other strand. Two single strands of DNA that come together by way of hydrogen bonding between stretches of complementary (or substantially complementary) nucleotides are said to be hybridized or annealed to one another. Because of the specificity of base-pairing, under most environmental conditions, a strand of DNA will have a specific stable complementary sequence.

31. This complementarity is critical for the heritability of genetic information. Because of the specificity of base-pairing, DNA polymerase can create or synthesize a complementary sequence from a single strand of a template sequence. As such, each strand of a double-stranded sequence can act as a template for the other. Cells are able to replicate DNA based on this property, and thus, pass exact copies of their genome to their progeny.

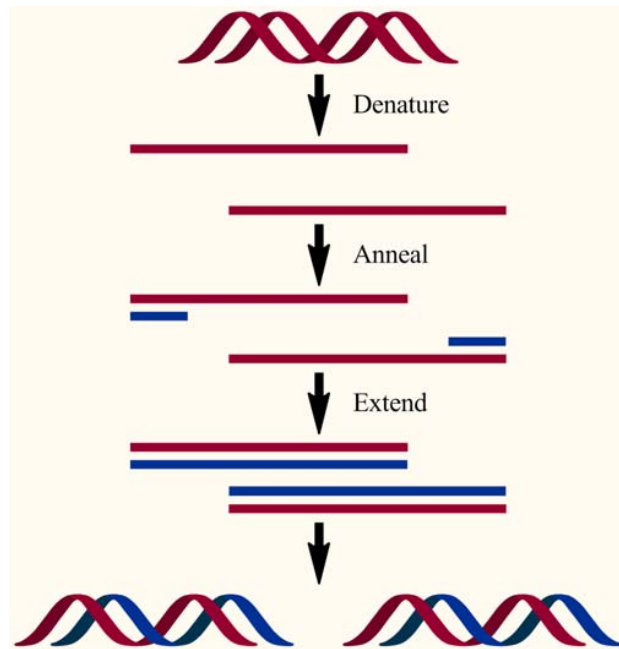
C. Amplification and detection

32. A universal technique for replicating or amplifying nucleic acids in a laboratory setting is the polymerase chain reaction, or just “PCR.” This method was invented by Dr. Kary Mullis in the 1980s. PCR is used to specifically amplify a small amount of nucleic acid molecules, generating thousands to millions of copies of the target nucleic acid. (Ex. 1018)(“Saiki 1985”)(Ex. 1019)(“Saiki 1988”).

33. Generally speaking, PCR involves the repeated denaturation and replication of DNA sequences in vitro (i.e., outside of an organism). DNA polymerase copies or synthesizes the complementary strand from a single-stranded template. For this enzymatic reaction to occur, a partially double-stranded section of DNA is required. Typically, a primer hybridizes to a complementary region of a single-stranded template. Dr. Mullis and his co-inventors used short oligonucleotide primers complementary to the 3'-ends of the complementary strands of a sequence of interest. Because of the base-pairing rules, primer sequences can be designed to

target specific nucleic acid sequences.

DNA polymerase initiates synthesis of the nascent strand from the hybridized primers in a 5'-to-3' direction to create double-stranded DNA. Two primers are used for each template strand in PCR that define the targeted sequence of interest. The general concept of PCR is illustrated by the diagram at right.



34. The first step of a PCR reaction is to melt or denature double-stranded DNA to yield single-stranded DNA. This typically is done by raising the reaction temperature to melt or by chemical means to separate double-stranded DNA or fragments into single strands. Following primer hybridization, the polymerase copies the template strand from the primer in a 5'-to-3' direction, making a complementary strand. Assuming optimal reaction conditions, the quantity of the original target sequence of interest, which is flanked by the two primers, will have been essentially doubled in one PCR cycle.

35. Next, the PCR cycle is repeated multiple times. This is accomplished by denaturing the double-stranded template, annealing the primers, and extending the primers with DNA polymerase to synthesize new double-stranded templates. At

each PCR cycle, the target sequence that lies between the primers (including the primer sequences themselves) will be replicated. Theoretically, a target sequence will be amplified exponentially as described by 2^n , where n is the number of cycles. In practice, however, the efficiency of the reaction varies, and decreases in later cycles. Regardless, PCR is able to create large quantities of DNA within a matter of hours.

36. PCR is a powerful tool for detecting human polymorphic variation that is associated with hereditary disease, aneuploidy, and cancer. Many techniques have been described to discriminate between wild-type and mutant alleles. For example, allele-specific PCR, also called competitive oligonucleotide priming (Ex. 1020)(“Gibbs 1989A”) or called amplification refractory mutation system (E. 1021)(“Newton”) has been used for detecting polymorphic-specific markers. Multiplex PCR allows for the simultaneous amplification of multiple target regions and has been used to detect coding exon deletion(s) in X-linked disorders (these exons are gene sequences that are transcribed into messenger RNA (mRNA) and translated into one or more proteins). X-linked disorders include Duchenne muscular dystrophy (Ex. 1022)(“Chamberlain”) and Lesch–Nyhan syndrome (Ex. 1023)(“Gibbs 1990”). Absence of specific PCR product(s) is diagnostic of exon deletion(s) in affected males, and ‘half-dosage’ PCR products are diagnostic of carrier mothers. Other detection methods that involve scanning (i.e., methods that

do not involve DNA sequencing) of PCR products for the presence of genetic alterations are summarized by a review by Dr. Markus Grompe. (Ex. 1024)(“Grompe”).

37. PCR can also be used to quantify the amount or the number of molecules of the target sequence in the biological sample. At least four basic methods have been described to quantify PCR-amplified templates: (i) quantitative PCR using an internal control, (ii) quantitative competitive PCR (Ex. 1025)(“Ferre”), (iii) real-time PCR (Ex. 1026)(“Heid”), and (iv) limited or end-point dilution PCR (Ex. 1027)(“Simmonds”)(Ex. 1028)(“Zhang”)(Ex. 1029)(“Lee”); (Ex. 1030)(“Sykes”)(Ex. 1031)(“Brisco”).

38. Real-time PCR using a 5'-exonuclease fluorogenic or TaqMan assay (Ex. 1032)(“Holland”) has been developed to accurately measure the starting amounts of target sequences or identify sequence variants, including SNPs. Unlike gel electrophoresis, real-time PCR has the unique advantage of being performed in a closed-tube system, which can significantly reduce carryover contamination. Using real-time PCR, one can easily monitor and quantitate the accumulation of PCR products during exponential or log phase amplification. The TaqMan assay utilizes dual reporter and quencher fluorescent dyes that are attached to a non-extendible probe sequence (i.e., the 3'-end of the probe is blocked). The reporter and quencher fluorescent dyes are attached at different internal positions or at

opposite ends to the oligonucleotide probe (Ex. 1033)(“Livak”) such that the fluorescent signal is quenched by the well-understood process called fluorescence resonance energy transfer (“FRET”). Quenched fluorescent probes are called fluorogenic probes. During the extension phase of PCR, the 5’-exonuclease activity of Taq DNA polymerase removes the nucleotides or digests the hybridized fluorogenic probe, which releases the reporter signal. The released reporter signal is measured during each cycle. In addition to quantifying nucleic acids, real-time PCR has broad utility for the identification of sequence variants, including SNPs.

39. The limiting or end-point dilution PCR method is based on diluting and distributing target sequences into individual reaction wells, such that the majority of wells has either zero or just one target sequence. The distribution of target sequences is based on Poisson statistics, which enables the mean number of target sequences per reaction well to be determined. Following the PCR amplification step, the reaction wells are analyzed to determine which reaction wells contain positive PCR products. The method has more recently been coined digital PCR. (Ex. 1034)(“Vogelstein”).

40. DNA sequencing includes several methods and technologies that are used to determine the order of nucleotide bases (A, C, G and T) in a given DNA fragment. DNA sequencing methods can generally be characterized as either first-generation sequencing, which include Sanger sequencing or next-generation

sequencing (“NGS”). In broad terms, NGS technologies are distinguishable from first-generation sequencing technologies in that they produce substantially larger volumes of sequence information cheaply. I have reviewed many of the platforms using NGS technologies. (Ex.1035)(“Metzker 2010”).

41. NGS technologies include a number of methods that are grouped broadly as template preparation, sequencing and imaging, and data analysis. NGS extends this process across millions of reactions in a massively parallel fashion, rather than being limited to one or a few nucleic acid fragments. Since their introduction, NGS platforms have allowed for high-throughput sequencing of nucleic acids on a genome-wide scale and highly multiplexed gene-specific sequencing.

42. The first NGS platform to achieve widespread commercial use was 454 Life Sciences Corporation’s sequencer, also known as Genome Sequencer 20 (“GS20”). (Ex. 1036)(“Marguiles”). The GS20 sequencer first became commercially available in 2005 and produced average read-lengths of ~100 bases. In 2007, 454 Life Sciences released its second instrument, called the GS FLX®, producing average read-lengths of ~250 bases. (Ex. 1037)(“Droege”). In that same year, 454 Life Sciences was acquired by Roche. Subsequently, the Roche/454 GS FLX® Titanium and GS FLX+ instruments have been released to produce longer average read-lengths.

43. The Roche/454 GS20, GS FLX® GS FLX® Titanium, and GS FLX+ sequencers all use a technology known as pyrosequencing, which is a method for detecting a single nucleotide incorporation event by a DNA polymerase as a bioluminescence signal. Specifically, nucleotide incorporation causes the release of a small molecule called pyrophosphate, which initiates a series of enzymatic reactions that produce the bioluminescence signal. Use of this technique typically requires shearing of the double-stranded genomic DNA into smaller pieces of DNA that can be sequenced, although PCR amplicons have been successfully used as templates. The Roche/454 sequencers then uses a bead-based immobilization strategy for DNA preparation. To create genomic libraries, the DNA is sheared into fragments of 300-800 base pairs, and two short pieces of additional DNA (called A/B adaptors) are attached to the ends of the fragmented genomic DNA. The A/B adaptors (or A/B primers) can also be attached to the 5'-ends of targeting primers to include these sequences in the final amplicons. A/B adaptors each consist of a known DNA sequences and provide priming sites for emulsion PCR and subsequently sequencing of the DNA fragments. The method, called emulsion PCR, is then used to copy individual DNA fragments onto beads, prior to the sequencing step.

44. Those beads are deposited onto a PicoTiterPlate® (“PTP”) that accommodates only one individual DNA-attached bead per well. Additional beads

coated with enzymes, called enzyme beads, are also added to the wells. Sequencing reagents (buffers, DNA polymerase, and nucleotides) are then flowed across the wells of the PTP. Nucleotides are sequentially added one at a time during a sequencing cycle. Nucleotide incorporation by the DNA polymerase causes release of pyrophosphate, which reacts with the enzyme beads and produces the bioluminescence signal. With the sequential flow of individual nucleotides, the timing of the bioluminescence signal indicates the order in which nucleotides are incorporated, which ultimately translates into the sequence of the DNA template.

45. The Genome Analyzer II (“GAII”) was developed by Solexa and released in late 2006. After the acquisition of Solexa by Illumina in 2007, this massively parallel sequencing platform achieved widespread commercial use. The GAII instrument utilizes a strategy called sequencing by synthesis (“SBS”), otherwise known as cyclic reversible termination (“CRT”), which was developed as an alternative to the Sanger sequencing method. To create genomic libraries, the DNA is sheared into smaller fragments, and short adaptors are attached or ligated to both ends of the DNA fragment. Bentley describes that the ligated products, which contain the adaptor sequences, are then PCR amplified to include additional sequences used in subsequent amplification and sequencing steps. (Ex. 1008)(“Bentley”). Solid-phase or bridge amplification is then used to make numerous copies of the DNA template. Following amplification, clusters are formed

on the flow cell that contain copies of the single original DNA template fragment. The result is that millions of template clusters are formed.

46. Following bridge amplification, the flow cells are prepared for the SBS or CRT sequencing approach. Then, a universal sequencing primer is added to the flow cell to hybridize to all templates amplified by bridge PCR. In the SBS approach, all four nucleotides contain a unique fluorescent label. Each nucleotide, however, is modified to act as a reversible terminator, which is an important feature of the technique. In the first step, all four reversible terminators are added simultaneously to the flow cells along with DNA polymerase. The DNA polymerase adds a single fluorescent reversible terminator that corresponds to the template base. The terminating group on the incorporated nucleotide blocks any other nucleotides from subsequently being incorporated. Following incorporation, the remaining unincorporated reversible terminators are washed away. Imaging is then performed to determine the identity of the incorporated nucleotide based on the fluorescent signal. This is followed by a cleavage step, which removes the terminating group and the fluorescent dye on the incorporated nucleotide. The cycle is repeated, and the fluorescent identity for each cluster is read using sequential images. Since 2009, Illumina has released new instruments including multiple versions of HiSeq®, MiSeq®, NextSeq® and NovaSeq® systems.

47. Other NGS platforms exist or have existed in the marketplace, for

example Life Technologies' SOLiD® instrument and Pacific Biosciences' single-molecule real-time or SMRT® instrument. (Ex. 1035). In 2011, Ion Torrent released their Personal Genome Machine or PGM®. The PGM® relies on the method of emulsion PCR to copy individual DNA fragments onto beads, called Ion Spheres®, which are deposited into an Ion Chip for sequencing. Sequencing primers and DNA polymerase are bound to the DNA fragments on the surface of the Ion Spheres, and these are loaded onto the chip in individual sensor wells, so that there is one bead per well. The four natural nucleotides (dATP, TTP, dGTP, dCTP) are dispensed one at a time across the Ion Chip. Sensors in each well detect a change in the pH when hydrogen ions are released by the incorporation of a nucleotide into a new growing primer. With the sequential flow of individual nucleotides, the timing of the pH change indicates the order in which nucleotides are incorporated, which ultimately translates into the sequence of the DNA template. (Ex. 1038)(“Rothberg”). Since 2011, Life Technologies has released the Ion Proton® with higher throughput of sequencing data compared with PGM®. More recently, Oxford Nanopore has released its MinION sequencer, which sequences individual DNA strands that are driven through tiny nanopores. (Ex. 1039)(“Jain”).

D. Creating a cell-free DNA library from maternal for aneuploidy detection was well known in the art

48. Prenatal diagnostics is an indispensable part of modern obstetrics care that targets fetal genetic diseases including chromosomal aneuploidy and single-

gene disorders. (Ex. 1040)(“Lo 2007A”). Definitive prenatal diagnosis requires the analysis of fetal genetic materials using invasive techniques such as amniocentesis or chorionic villus sampling (“CVS”). These invasive procedures carry a finite risk of fetal loss, and many researchers have searched over decades for non-invasive alternatives to sample fetal genetic materials. One early approach was to isolate nucleated fetal cells from maternal blood, though because of the extreme rarity of these cells – basically one cell in each milliliter of maternal blood – these heroic efforts proved intractable for routine usage.

49. Not all DNA or RNA, however, is isolated from cellular materials. In 1997, Dr. Dennis Lo made the remarkable discovery that cell-free fetal DNA could be isolated and characterized from plasma or serum obtained from pregnant mothers. (Ex. 1041)(“Lo 1997”). Lo 1997 describes that the plasma- or serum-based approach might also be applicable to screening for chromosomal aneuploidies, such as Down syndrome. By 2000, Dr. Lo also discovered that cell-free fetal RNA could be isolated and characterized from plasma obtained from pregnant mothers. (Ex. 1042)(“Poon”). Cell-free DNA that isolated from maternal blood is composed of a mixture of both maternal and fetal nucleic acids. By 2007, much had been learned about the biology of cell-free DNA in maternal blood including that cell-free fetal DNA (i) consists of primarily short fragment lengths (the majority shorter than 200 bp), (ii) represents a small subfraction of the total cell-free DNA isolated in maternal

blood, (iii) increases in concentration during gestation, and (iv) comes from normal cell turnover events, such as apoptosis and necrosis, of the placenta, which is the predominant source of fetal DNA in maternal plasma.

50. The discovery of cell-free fetal nucleic acids in maternal plasma launched a new industry of non-invasive prenatal diagnostic approaches. The first analytical methods focused on ways to differentiate fetal DNA from the overwhelming amount of maternal DNA in plasma. This was accomplished by targeting specific paternal chromosomal regions, genes, or genetic variations that were inherited by the fetus from the father and were absent in the mother. These applications included detecting Y chromosome sequences in male fetuses and rhesus blood group antigen D (“RHD”) positive fetuses in the plasma of RHD-negative mothers. Other approaches to detect cell-free fetal nucleic acids focused on markers of the placenta, which is of fetal origin, that either were expressed as a specific DNA methylation pattern or as specific RNA molecules.

51. In 2007, two papers were published contemporaneously that described the use of digital PCR for the detection of chromosomal aneuploidy, specifically related to trisomy 21 or Down syndrome. (Ex. 1043)(“Lo 2007B”)(Ex. 1044)(“Fan 2007”). Unlike previous efforts, Lo 2007B and Fan 2007 both described the use of digital PCR to count the number of positive products from PCR targets that did not rely on differences between fetal and maternal DNA. The challenge with this non-

polymorphic approach was that method must be able to quantitatively measure small differences in an aneuploidy sample compared with a normal sample. As described above, fetal DNA represents only a small fraction (on average, ~10%) of the total cell-free DNA isolated in maternal plasma. In the example where 100 copies of a genomic region derived chromosome 21 were isolated from one milliliter of plasma, 90 copies would be maternal and 10 copies would be fetal. For a trisomy 21 pregnancy, the maternal copies would be the same, but the fetus would contribute 15 copies, whereby 105 copies of total cell-free DNA would be isolated from one milliliter of plasma. In other words, the sensitivity of digital PCR in this example would need to be high enough to correctly characterize 105 copies as those derived from an aneuploidy fetus compared with 100 copies from an euploidy fetus.

52. This non-polymorphic approach relies simply on counting the number of PCR products from a sequence region suspected of having a chromosomal aneuploidy and a second sequence region suspected being normal or euploidy. These results would then be compared to a normal sample, whereby the sample having chromosomal aneuploidy could be identified with statistical significance. The precision of digital PCR to accurately measure the small differences in cell-free copy numbers is directly related to (i) percentage of fetal DNA in the cell-free DNA fraction isolated from maternal plasma and (ii) the number of PCR reactions performed for a given assay. For example, Lo 2007B estimated that it would require

7,680 digital PCR reactions to correctly classify trisomy 21 samples in 97% of cases that contained a fetal fraction of 25% from cell-free DNA isolated from maternal plasma. With lower fetal fractions, such as 10%, a much higher number of reaction samples would need to be performed to maintain the level of precision estimated by Lo2007B.

53. As described above, NGS technologies produces large numbers of sequence read data. In 2008, the same research groups led by Dr. Dennis Lo and Dr. Stephen Quake described the application of NGS to chromosomal aneuploidy detection in pregnant mothers. (Ex. 1013)(“Chiu”)(Ex. 1006)(“Fan 2008”). NGS provides much deeper data sampling than that of digital PCR. Both groups described generating several million sequence reads from the cell-free DNA isolated from maternal plasma and mapping or aligning those reads to the human reference genome. The number of mapped sequence reads were then counted to determine the over- and underrepresentation of each chromosome contributed by the fetus. Of the 28 cases analyzed by Chiu, 14 trisomy 21 (Down syndrome) fetuses and 14 euploidy fetuses were correctly identified. Of the 18 cases analyzed by Fan 2008, nine trisomy 21, two trisomy 18 (Edwards syndrome), one trisomy 13 (Patau syndrome) fetuses and six euploidy fetuses were correctly identified.

54. To perform NGS, sequencing libraries were often created from samples using PCR amplification. PCR-based library preparation was well-known and

widely-used by the time the first application leading to the '831 Patent was filed. (Ex. 1012, pp. 001-002)(Ex. 1006, p. 003, right column)(Ex. 1007, p. 002, right column)(Ex. 1014, p. 001-002)(Ex. 1003, ¶¶0160-0161). By that time, in fact, standard kits to prepare sequencing libraries had been available for at least around two years. (Ex. 1014, p. 001)(Ex. 1005, 3:32-4:2)(Ex. 1003, ¶¶00179)(Ex. 1007, p. 002, right column). These kits, like the recited claims, employed PCR amplification and utilized primers with sample specific barcodes and sequences to which the sequencing primer could anneal. The kits were to be used with next-generation sequencers which rendered use of such sequences a necessity for fast and cheap sequencing.

55. Next generation sequencers could sequence millions of DNA molecules in a single run and thereby made feasible sequencing DNA from different individuals at the same time. To distinguish samples from different individuals, DNA from a particular individual was “tagged” or “indexed” such that each individual sample had a separate tag, index or barcode. Thus, a “tagged” or “indexed” library of that individual’s DNA fragments could be created and the “tag” or “index” could be used to associate a particular DNA with an individual. The advantage of “indexing” was that DNA from different individuals could be pooled and sequenced together in a single reaction.

56. Not only was the concept of creating an indexed DNA sequencing

library routine and commonplace, the concept of using cell-free DNA from maternal blood to detect chromosomal aneuploidy, such as trisomy 21, the underlying cause of Down syndrome was equally well-known in the art. (Ex. 1003, ¶0122)(Ex. 1005, 1:31-2:5)(Ex. 1006, Abstract). Cell-free DNA in maternal DNA was discovered more than a decade before the priority date and triggered an immediate interest in developing non-invasive prenatal diagnostics. This was because fetal abnormalities could be tested using maternal blood, rather than having to undergo invasive ultrasound procedure with its attendant risk.

57. Soon after the discovery of cell-free DNA, scientists were analyzing the cell-free DNA for fetal abnormalities including aneuploidies. Some of the more common aneuploidies being tested included trisomy (where an individual has three copies of a particular chromosome) of chromosomes 13 (Patau syndrome), 18 (Edwards syndrome) and 21 (Down syndrome). A common way to test for aneuploidy was to sequence the chromosome of interest and use statistical analysis to determine whether the amount of sequences from that chromosome were unusually high or low. (Ex. 1006, p. 001)(Ex. 1013, p. 001).

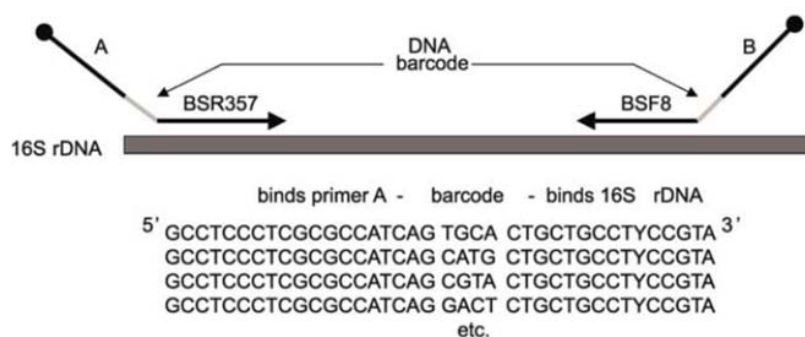
E. Use of indexed DNA library for multiplexed next generation sequencing was wide spread in the art

58. As described above, NGS technologies produce substantially larger volumes of sequence information cheaply in a single run. There are many sample types that can be analyzed using NGS technologies including, but not limited to,

human and other mammalian genomes and transcriptomes, targeted-enrichment of exons or chromosomal regions of these genomes, lower eukaryotic and prokaryotic genomes, and metagenomic samples. In many cases, the NGS instrument throughput far exceeds the sequencing needs of a single sample. To overcome this inefficiency, samples are multiplexed, which means that multiple samples are analyzed by NGS technologies in a single run. This is accomplished by two strategies: (i) physical separation of multiple samples within the Roche/454 PTP or Illumina 8-channel flow cell and/or (ii) molecular barcodes that associate individual DNA fragments with a given sample. For example, samples obtained from a given microbial source have been widely analyzed by the Roche/454 pyrosequencing instruments using barcoded PCR amplicons of 16S rRNA sequences. (Ex. 1045)(“Binladen”); (Ex. 1046)(“Huber”); (Ex. 1047)(McKenna”); (Ex. 1048)(“Hamady 2008”); (Ex. 1049)(“Andersson”); (Ex. 1050)(“Fierer”); (Ex. 1051)(“Dethlefsen”); and (Ex. 1052)(“Hamady 2009”).

59. In addition to the term barcode, these molecular codes have been referred to as tags, multiplex identifiers, and indexes. A barcode consists of a short DNA sequence. The number of nucleotides in a given barcode sequence can range from 3-12 bases and are empirically designed to minimize artifacts due to non-equal representation of particular barcode sequence in the sequencing data or to conform to error profiles of the sequencing method. (Ex. 1053)(“Parameswaran”).

60. Barcoded primers consist of three regions that serves three functions. For example, for the Roche/454 pyrosequencing method, barcode sequences are generally inserted between the A/B primers, used for emulsion PCR and sequencing processes, and the target specific primer region (i.e., 16S rDNA). The barcoded primers are simply made using automated oligonucleotide synthesis machines. An example of McKenna's barcoded primers are illustrated below.



(Ex. 1047, p. 0004, Figure 1B).

61. McKenna's barcoded primers have the general structure of Primer A–Barcode–16S rDNA primer. Primer A, which is common to all barcoded primers of the set, serves the function to prime the emulsion PCR and sequencing steps of the Roche/454 system. The Barcode, which is specific for each barcoded primers of the set (i.e., “TGCA,” “CATG,” etc.), serves the function to uniquely tag a given sample that can be later identified by sequence analysis. 16S rDNA primer, which is also

common to all barcoded primers of the set, serves the function to prime target specific 16S rDNA amplification of each sample during the PCR process.

62. As of January 2010, Illumina's GA sequencer was the most commonly used next-generation sequencer. As I explain earlier, the workflow of Illumina's GA sequencer generally consisted of four steps: DNA library preparation, cluster generation, sequencing-by-synthesis on the GA instrument, and data analysis. (Ex. 1008, p. 001-002)(Ex. 1012 at 001-002, Figure 1). The GA instrument was capable of sequencing a sample from an individual or multiple samples from many different individuals.

63. For example, Bentley used the GA analyzer to analyze an individual human genome. (Ex. 1008, p. 001). To create the DNA library, human genomic DNA was sheared into different fragments. (*Id.*). Two adaptor sequences were ligated to both ends of the DNA fragments. (*Id.*, p. 008). These adaptor-ligated fragments were amplified using two PCR primers targeting the two adaptor sequences, respectively. (*Id.*, pp. 008-009). These PCR primers "include the sequences that anneal to the complementary oligonucleotides bound to the flowcell surface, plus the sequencing primer sites." (*Id.*, p. 009) As shown in the graphic below (created by me), for the paired-read experiments, the two adaptor sequences contain sequences (highlighted in yellow and teal, respectively), which hybridize to their respective two PCR primers and the sequencing primers. (*Id.*) Because all of

the amplified DNA fragments have the same sequencing primer sites, they can all be sequenced using the same pair of sequencing primers.

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Adaptor 1:                               5'ACACTCTTTCCCTACACGACGCTCTTCCGATCXT
PCR Primer 1: 5'-CAAGCAGAAGACGGCATACGAGATCGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCXT
Rd1 Sequencing Primer:                   5'-ACACTCTTTCCCTACACGACGCTCTTCCGATCT-3'
=====
Adaptor 2:                               5'-phosphate-GATCGGAAGAGCGGTTCAGCAGGAATGCCGAG
PCR Primer 2: 5'-AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCGATCXT
Rd2 Sequencing Primer:                   5'-CGGTCTCGGCATTCTGCTGAACCGCTCTTCCGATCT-3'

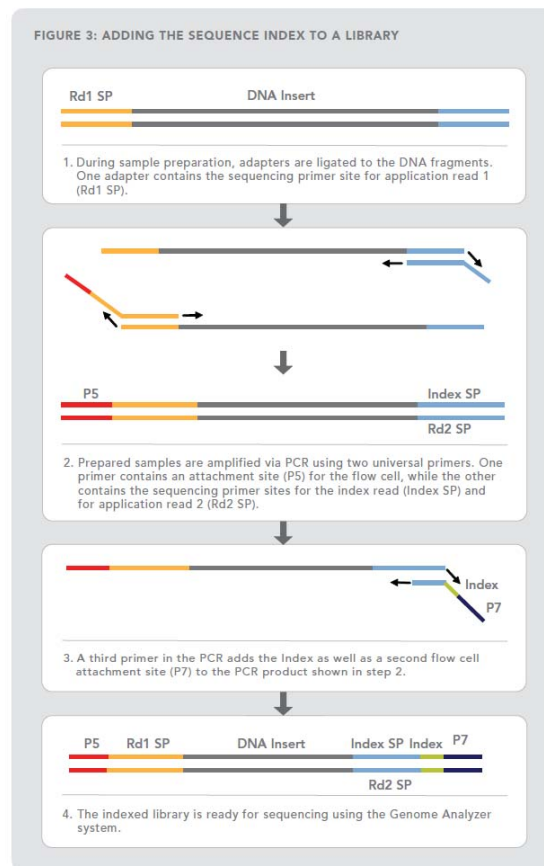
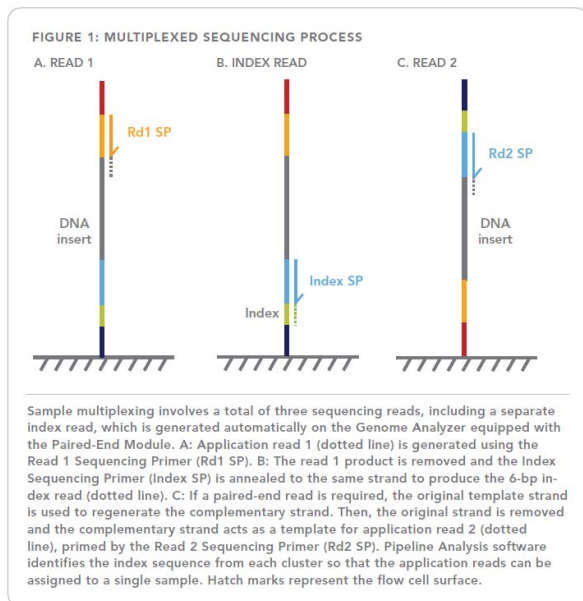
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64. In addition, Quail describes improvements to Illumina's sequencing system, and discusses the use of adaptors "which essentially consist of the sequences to which the sequence primers hybridize during the sequencing reaction." (Ex. 1012, p. 002)("Quail"). It also discusses introducing these adaptors during a PCR amplification step. (*Id.*, p. 003)("The PCR step introduces into the adapter-ligated template molecules the oligonucleotide sequences required for hybridization to the flowcell surface."). As with Bentley, because the DNA fragments in Quail have the same sequencing primer sites ("the sequences to which the sequence primers hybridize"), they can all be sequenced using the same pair of sequencing primers.

65. Moreover, as of January 2010, Illumina was selling library preparation kits for multiplex sequencing on its GA sequencer. (Exs. 1014, 1016). To perform multiplexed sequencing on the GA sequencer, a DNA library from each sample is

typically prepared as follows. (Ex. 1014, pp. 002-003, Figure 3)(Ex. 1016). First, two adaptor sequences are ligated to both ends of the DNA fragments in each sample. The ligated products are then amplified using two universal primers targeting those adaptor sequences and are tagged with a unique “index” sequence using a third PCR primer. (*Id.*). After library preparation, multiple libraries are pooled and sequenced simultaneously on the Genome Analyzer.

66. As shown below (Exhibit 1014, Figs. 1 and 3), these adaptors serve several functions. The adaptors contain sequences that hybridize to (i) universal primers for PCR amplification and (ii) their respective sequencing primers for sequencing the target DNA fragments. Thus, they are also called “sequencing adaptors.” One of the adaptors further contains sequences that hybridize to (iii) a third PCR primer to add the index sequence and (iv) a third sequencing primer for sequencing the index. (Ex. 1016, p. 003)(“The adapters contain sequences that correspond to the two surface-bound amplification primers on the flow cells used in the Cluster Station. One of the also contains the index sequence.”)(*id.*, Figs. 1 and 2 (workflows), 014 (adaptors)). Thus, the amplified DNA fragments can all be sequenced using the same sequencing primers on the Genome Analyzer.



67. Craig (Ex. 1056) described a generalized framework for multiplex resequencing of targeted genome regions on the Illumina GA and GAII instruments. Here, 46 HapMap individuals, previously characterized by the Encyclopedia of DNA Elements (“ENCODE”) project, were evaluated by the targeted resequencing method. Here, Craig amplified the 46 HapMap individuals by whole genome amplification followed by two long-range amplification steps using PCR that 24 regions across the human genome. For each HapMap individual, the 5-kb amplicons for the 14 regions were then pooled together in equal portions and digested with DNase I enzyme to produce fragments in the size range of 200-300 bp. Following

additional library steps, a unique indexed-adaptor sequences was ligated to each HapMap individual library sample by an enzyme called DNA ligase. As described above in this declaration, indexed sequences were routinely added by PCR as well. For example, Illumina described adding index sequences to its adaptor-ligated DNA fragments using a PCR enrichment step. Following additional library steps, each HapMap sample was pooled into a single tube and subjected to a PCR enrichment step using Illumina compatible primers. The pooled indexed-library of 46 HapMap individuals were then sequenced using either an Illumina GA or GAI instrument. Craig reported that >90% of genetic variants were discoverable using their targeted multiplex sequencing approach.

F. By 2010, skilled artisans were focused on creating target-specific DNA libraries

68. As described in my 2010 review of the NGS field, “[d]espite the substantial cost reductions associated with NGS technologies in comparison with the automated Sanger method, whole-genome sequencing is still an expensive endeavor. An interim solution to this problem may be to use NGS platforms to target specific regions of interest.”

69. The concept of targeting specific regions of the genome is well established, with PCR being the most widely used method, albeit on a small scale. A strategy based on microdroplet PCR technology was developed in 2009 to simultaneously amplification of 3,976 products. Here, a microfluidic device creates

aqueous picoliter volume droplets of targeting forward and reverse primers in an oil solution. Primer droplets targeting different regions merge with separate picoliter droplets containing fragmented barcoded genomic DNA and associated PCR reagents, which are then thermal cycled in a single tube. The authors reported an 84% capture efficiency with 90% of the targeted bases showing uniform coverage using the microdroplet PCR method sequenced with either Roche/454 or Illumina/Solexa platform. (Ex. 1054)(“Tewhey”).

VI. BASIS OF MY OPINION

70. I understand that the '831 Patent (Ex. 1001) was filed on April 2, 2015 and claims an earliest priority to January 23, 2010, which is the filing date of U.S. Provisional Serial No. 61/297,755 (the “'755 Provisional,” Ex. 1055). I have not seen any evidence to suggest that any claim of the '831 Patent is entitled to the benefit of an earlier priority date. I am not aware of any claim by the Patent Owner to an earlier priority date that would change any of my opinions as set forth herein. For my analysis below, I have considered January 23, 2010 as the relevant date.

71. In preparing this Declaration, I have reviewed the '831 Patent (Ex. 1001), its file history (Ex. 1010) and the '755 Provisional (Ex. 1055), and considered each of the documents cited herein, in light of general knowledge in the art before January 23, 2010.

72. In formulating my opinions, I have relied upon my 30+ years of

education, training, knowledge, and experience in the relevant art. But at all times, I have attempted to understand and interpret the claims of the Patents-in-Suit from the point of view of the person of ordinary skill in the art as of the early January, 2010 time period.

73. I understand that for the purposes of an IPR, claims are to be given their broadest reasonable interpretation (“BRI”) in light of the specification of the ’831 Patent.

VII. THE ’831 PATENT

74. I understand that the ’831 Patent (Ex. 1001) resulted from the ’854 Application, filed on April 2, 2015, which claims earliest priority to the ’755 Provisional filed on January 23, 2010 and is entitled “methods of fetal abnormality detection.” (Ex. 1055).

75. I have been informed by counsel for Petitioner that claims 1 and 14 are independent claims, which set forth the elements of the invention and do not rely upon or refer to any other claims in the patent.

76. I have been informed by counsel for Petitioner that claims 2-10, 12-13, 15-22 and 24 are dependent claims, which contain all of the features of the claim from which they depend but also contain additional features or limitations. In this case, claims 2-10 and 12-13 are dependent from claim 1 since they refer directly to claim 1, while claims 15-22 and 24 are dependent from claim 14 since they refer

directly to claim 14.

VIII. RELEVANT LAW

77. I am not a lawyer, but I understand the following concerning the applicable law:

A. Anticipation

78. I understand that a patent claim is anticipated when a single piece of prior art discloses every element of the claimed invention, either expressly or inherently, arranged in the same way as in the claim. For inherent anticipation to be found, it is required that the missing descriptive material is necessarily present in the prior art. I understand that, for the purpose of an *Inter Partes* review, prior art that anticipates a claim can include both patents and printed publications from anywhere in the world.

B. Obviousness

79. I understand that a claim in an issued patent can be unpatentable if it is obvious. Unlike anticipation, obviousness does not require that every element of the claim be in a single prior art reference. Instead, it is possible for claim elements to be described in different prior art references, so long as there is motivation or sufficient reasoning to combine the references and a reasonable expectation of success. The prior art references must also be “analogous art,” meaning that they are either from the same field of endeavor as the ’831 Patent, regardless of the problem addressed, or reasonably pertinent to the particular problem with which the

inventor is involved.

80. I understand that a claim is unpatentable for obviousness if the differences between the claimed subject matter and the prior art are such that the subject matter as a whole would have been obvious at the time the alleged invention was made to a person having ordinary skill in the art to which said subject matter pertains.

81. I understand, therefore, that when evaluating obviousness, one must consider obviousness of the claim “as a whole.” This consideration must be from the perspective of one of ordinary skill in the relevant art, and that such perspective must be considered as of the “time the invention was made.”

82. The level of ordinary skill in the art is discussed in ¶79 below.

83. The relevant time frame for obviousness, the “time the invention was made,” is discussed in ¶91, below.

84. I understand that in considering the obviousness of a claim, one must consider the so-called *Graham* factors, including the following four things. These include the scope and content of the prior art, the level of ordinary skill in the art at the relevant time, the differences between the prior art and the claim, and any “secondary considerations.”

85. I understand that “secondary considerations” include real-world evidence that can tend to make a conclusion of obviousness either more probable or

less probable. For example, the commercial success of a product embodying a claim of the patent could provide evidence tending to show that the claimed invention is not obvious. In order to understand the strength of the evidence, one would want to know whether the commercial success is traceable to a certain aspect of the claim not disclosed in a single prior art reference (i.e., whether there is a causal “nexus” to the claim language). One would also want to know how the market reacted to disclosure of the invention, and whether commercial success might be traceable to things other than innovation, for example the market power of the seller, an advertising campaign, or the existence of a complex system having many features beyond the claims that might be desirable to a consumer. One would also want to know how the product compared to similar products not embodying the claim. I understand that commercial success evidence should be reasonably commensurate with the scope of the claim, but that it is not necessary for a commercial product to embody everything that would fall within the scope of the claim.

86. Other kinds of secondary considerations are possible. For example, evidence that the relevant field had a long-established, unsolved problem or need that was later provided by the claimed invention could be indicative of non-obviousness. Evidence that others had tried but failed to make an aspect of the claim might indicate that the art lacked the requisite skill to do so. Evidence of copying of the patent owner’s products before the patent was published might also indicate that

its approach to solving a particular problem was not obvious. Evidence that the art recognized the value of products embodying a claim, for example, by praising the named inventors' work, might tend to show that the claim was non-obvious.

87. I further understand that prior art references can be combined where there is an express or implied rationale to do so. Such a rationale might include an expected advantage to be obtained or might be implied under the circumstances. For example, a claim is likely obvious if design needs or market pressures existing in the prior art make it natural for one or more known components to be combined, where each component continues to function in the expected manner when combined (i.e., when there are no unpredictable results). A claim is also likely unpatentable where it is the combination of a known base system with a known technique that can be applied to the base system without an unpredictable result. In these cases, the combination must be within the capabilities of one of ordinary skill in the art.

88. I understand that when considering obviousness, one must not refer to teachings in the specification of the patent itself. One can, however, refer to portions of the specification admitted to being prior art, including the "BACKGROUND" section. Furthermore, a lack of discussion in the patent specification concerning how to implement a disclosed technique can support an inference that the ability to implement the technique was within the ordinary skill in the prior art.

89. I also understand that, to combine or modify a reference in a way that

can be called obvious, one of ordinary skill would have to have a reasonable expectation of success. I understand that the reasonable expectation of success does not mean certainty or a guarantee of success, but rather only that it is a “reasonable” expectation. The reasonable expectation of success is required for what is recited in the claims but is not required for concepts not recited in the claims.

IX. LEVEL OF ORDINARY SKILL IN THE ART

90. In my opinion, the relevant art was that of molecular biology. I have considered the level of ordinary skill based on my experience in the field, and the educational level of the inventor; the type of problems encountered in the art; prior art solutions to those problems; rapidity with which innovations are made; sophistication of the technology; and educational level of active workers in the field. I understand that not all such factors may be present in every case, and one or more of these or other factors may predominate in this case. In the present case, I understand that both inventors were Ph.D.-level molecular biologists, and that in 2010, Dr. Chuu had approximately 15 years of experience, while Dr. Rava had almost 30 years of experience. Based on this and on my experience in working with others in the field, I believe that the ordinary artisan in the relevant timeframe was highly skilled compared to many other industries and would have had a Ph.D. in molecular biology or a related field and approximately five years of experience working with or researching genetic testing, DNA amplification, and DNA

sequencing.

X. RELEVANT TIMEFRAME FOR DETERMINING OBVIOUSNESS

91. I understand that obviousness must be evaluated “at the time of the invention.” From the cover page of the ’831 Patent, the ’755 Provisional was filed in the United States on January 23, 2010. For the purpose of this declaration, I will analyze obviousness in the time frame immediately prior to this date, although my testimony is usually applicable to a longer period of time before January 23, 2010. My testimony is directed to this timeframe, unless otherwise indicated, even if I do not always use a past tense.

XI. CLAIM CONSTRUCTION

A. Claims 1, 6, 7, 14, 18 and 9—“Aneuploid”

92. I have reviewed the construction of the term “aneuploid” in the petition for *Inter Partes* review, and I agree based on the specification at Ex. 1001, 13:32-46 that “aneuploid” should be defined as “having an abnormal number of chromosome(s), or parts of a chromosome.”

I. GROUNDS OF UNPATENTABILITY

Ground 1. Claims 1-7, 10, 14-19, and 22 are obvious over Fluidigm in view of the knowledge of one of ordinary skill

93. It is my opinion that claims 1-7, 10, 14-19, and 22 of the ’831 Patent would have been obvious to one of ordinary skill in the art in the relevant timeframe over U.S. Pat. App. Publication 2010/0120038 (“**Fluidigm**”) in view of the

knowledge of a person of ordinary skill, as reflected in, for example:

- Ex. 1006, Fan, et al., “Noninvasive diagnosis of fetal aneuploidy by shotgun sequencing DNA from maternal blood,” Vol. 105:16266–16271 PNAS (October 21, 2008)(“**Fan 2008**”);
- Ex. 1008, Bentley, et al., “Accurate whole human genome sequencing using reversible terminator chemistry,” Nature, Vol 456:53-59 (November 2008)(“**Bentley**”);
- Ex. 1012, Quail, et al., “A large genome center’s improvements to the Illumina sequencing system,” Nature Methods, Vol. 5:1005-1010 (Dec. 2008)(“**Quail**”);
- Ex. 1013, Chiu, et al. “Noninvasive Prenatal Diagnosis of Fetal Chromosomal Aneuploidy by Massively Parallel Genomic Sequencing of DNA in Maternal Plasma,” PNAS Vol. 105:20458-20563 (Dec. 23, 2008)(“**Chiu**”);
- Ex. 1014, “Multiplexed Sequencing with the Illumina Genome Analyzer System”, Illumina, 2008 (“**Illumina Datasheet**”).

Overview of Fluidigm

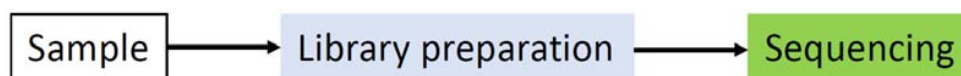
94. The Fluidigm publication discusses methods for improving preparation of DNA libraries, and in particular libraries to be used for later sequencing. Such sequencing libraries are typically amplified target DNA sequences that can have adaptors and index sequences added. Fluidigm discusses library preparation, for

example, near the beginning of its disclosure:

“The present invention relates generally to the area of high-throughput assays for detection of particular target nucleic acids. In particular, the invention relates to **methods for increasing the number of samples and/or targets** that can be analyzed in a single assay.”

(Ex. 1003, ¶0002)(Emphasis added)(Ex. 1004, ¶0002). I understand that the phrase “methods for increasing the number of samples and/or targets that can be analyzed” to be steps of library preparation. The libraries prepared can be used for “assays,” as Fluidigm states, which would include sequencing to determine the existence or amount of particular DNA sequences. For example, Fluidigm states: “[i]f desired, **tagged target nucleotide sequences generated as described herein** may be analyzed by **DNA sequencing**.” (Ex. 1003, ¶0160)(Emphases added)(Ex. 1004, ¶0135).

95. In this way, Fluidigm discloses teaches preparation of a DNA sequence library, followed by sequencing of that library, as shown in the following diagram (drawn by the Petitioner):



96. Fluidigm has several methods to create sequencing libraries. These

methods involve various amplification and tagging steps. One method (the so-called “Modular Approach” is very close to anticipating the claims, as I understand anticipation (see ¶77, above). The “Modular Approach” is discussed mainly under the heading “Detection of Multiple Target Nucleic Acids—Modular Approach” beginning in paragraph 0115 of Fluidigm, although disclosure throughout Fluidigm modifies the Fluidigm embodiments where indicated. The Modular Approach uses three targeted amplification steps on which the steps of independent claims 1 and 14 read. (Ex. 1003, ¶¶0119-0120)(Ex. 1004, ¶¶0094-0095).

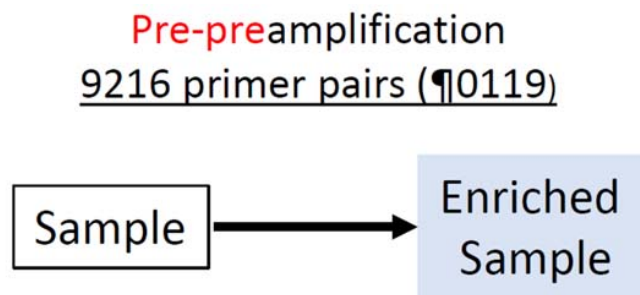
97. The Modular Approach of Fluidigm begins with a sample. The sample can be a sample of maternal blood containing fetal DNA being tested for aneuploidy. (Ex. 1003, ¶¶0122, 0175)(Ex. 1004, ¶¶0097, 0150). It would have obvious to one of ordinary skill in the art the obtaining a maternal blood sample would contain both maternal and fetal cell-free DNA based on the teachings of Lo 1997 (Ex. 1041), Lo 2007A (Ex. 1040), Lo 2007B (Ex. 1043), Fan 2007 (Ex. 1044), Chiu (Ex. 1013), and Fan 2008 (Ex. 1006), for example.

98. The first step of the Modular Approach is to use a “**pre-pre-amplification**” step. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The pre-pre-amplification step uses PCR amplification with 9,216 primer pairs, to amplify 9,216 target sequences. For example, Fluidigm discloses:

“To increase target nucleic acid concentration prior to

encoding, an optional pre-preamplification reaction can be carried out before the encoding preamplification reaction. The pre-preamplification can be carried out in multiplex [sic: multiplex]. For example, target-specific primers for 9216 different target nucleic acids can be employed in one mixture.”

(Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The method thus far can be depicted as follows:

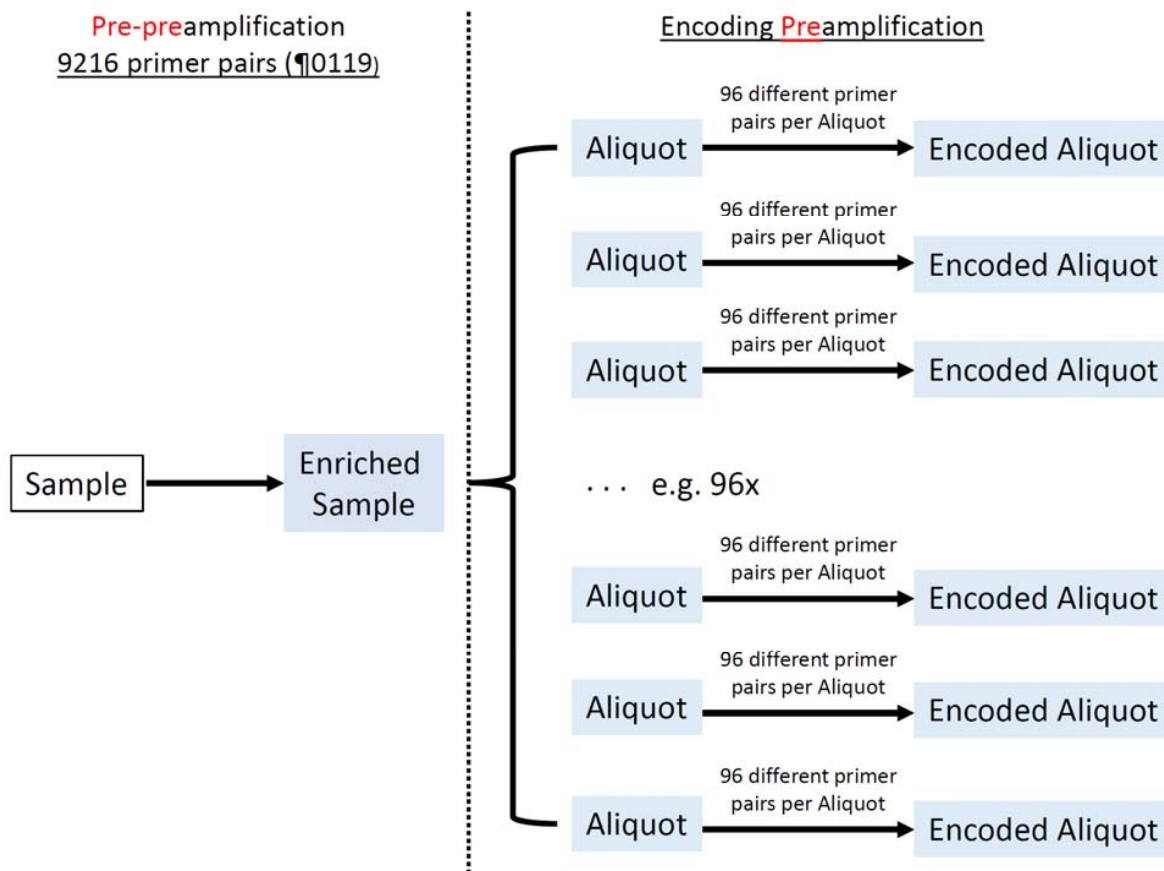


99. The second amplification step in the Fluidigm Modular Approach is the “encoding pre-amplification” step. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). In this step, the output of the first step is divided into different aliquots (in Fluidigm, the example of 96 aliquots is used). (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The 96 aliquots are then each subjected to PCR amplification, using 96 different primer pairs in each aliquot. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The 96 primer pairs per aliquot can be used to introduce “nucleotide tag pairs.” (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The nucleotide tag pairs, which are also known as “bar codes” or “encoding sequences”, are sequences whose purpose can be to identify the DNA sequences in

the sample, or to identify different samples. (Ex. 1003, ¶¶0064, 0094, 0115)(Ex. 1004, ¶¶0005-0008, 0071, 0077-0078, 0082). Fluidigm describes the “encoding pre-amplification” step immediately after describing the “pre-pre-amplification step”, as follows:

“The **pre-preamplification** can be carried out in multiplex [sic: multiplex]. For example, target-specific primers for 9216 different target nucleic acids can be employed in one mixture. **This mixture can then be divided into R=96 aliquots and each aliquot subjected to an encoding preamplification reaction** on a microfluidic device, using T=96 different primer pairs that add 96 different nucleotide tag pairs to the target nucleotide sequences in each of the 96 aliquots.”

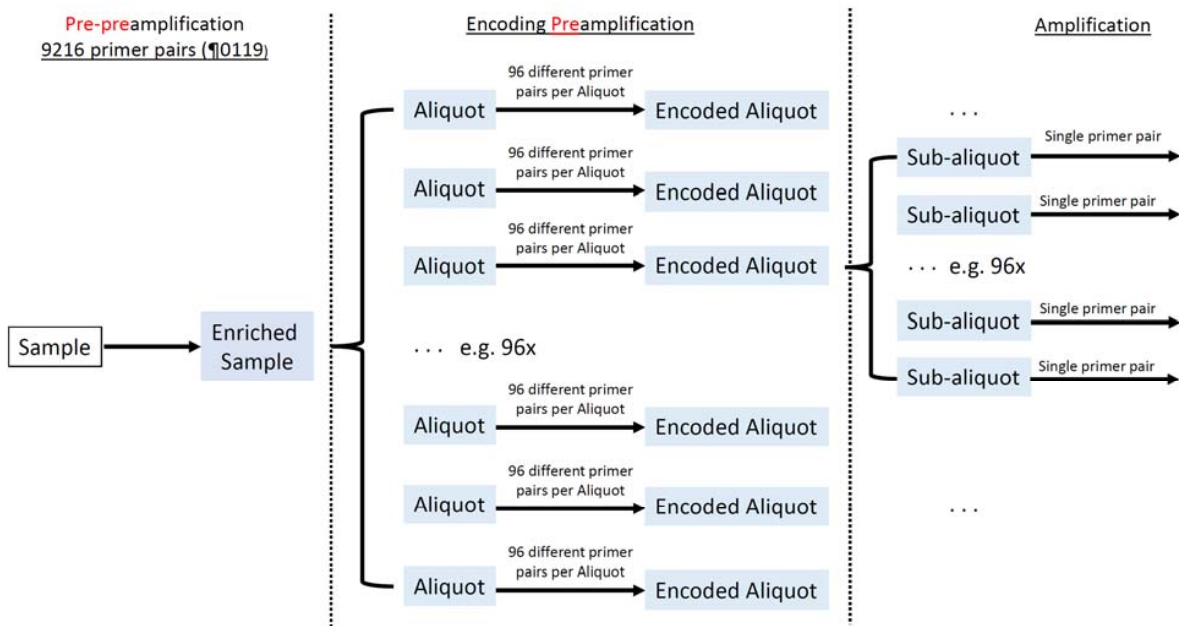
(Ex. 1003, ¶0119)(Emphases added)(Ex. 1004, ¶0094). After the “encoding pre-amplification” step, the Fluidigm Modular Approach can be depicted as follows (illustration by Petitioner):



100. Following the second amplification (*i.e.* the “encoding preamplification”) step, each of the separate aliquots containing tagged DNA proceeds to separate chambers in a microfluidic device. (Ex. 1003, ¶0120)(Ex. 1004, ¶0095). There, each aliquot will be subdivided 96 times. Each sub-aliquot is subjected to a third amplification using a single PCR primer pair. (Ex. 1003, ¶0120)(Ex. 1004, ¶0095). The single primer used with each subaliquot is specific to the combination of sequence tags used in the “encoding preamplification” step. (Ex. 1003, ¶0120)(Ex. 1004, ¶0095). For example, Fluidigm discloses:

“After the encoding preamplification reaction, amplification can be carried out in separate chambers of a microfluidic device. For example, each of the 96 aliquots produced upon encoding preamplification can be loaded into individual sample lines of a matrix-type micro fluidic device, and each of 96 different tag-specific primer combinations can be loaded into individual assay columns. Each different of the 96 primer combination can amplify a different target nucleic acid in each of the 96 aliquots. The resulting 9216 reaction chambers (sub-aliquots) can then be subjected to amplification, followed by detection of amplification product(s)....”

(Ex. 1003, ¶0120)(Emphasis added)(Ex. 1004, ¶0095). I understand this passage to mean that each subaliquot is targeting a different sequence. However, the primers are targeted to specific tag combinations introduced in the encoding amplification. By targeting tag combinations, fewer primers overall are needed for the third amplification step. By physically separating subaliquots, the tag sequences can be re-used, and there is an increased chance of having a single, specific PCR product with minimal to no non-specific products being formed (only one primer pair should be present with each sub-aliquot). With the addition of this amplification step, the Fluidigm Modular Approach can be depicted as follows (illustration by Petitioner):



101. Although Fluidigm teaches that each encoded aliquot is subdivided, the diagram above shows only a single subdivision to save space. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The output of the entire Modular Approach includes 9,216 separate sub-aliquots with 9,216 enriched, tagged DNA sequences to create a sequencing library. (Ex. 1003, ¶¶0160, 0177)(Ex. 1004, ¶¶0135, 0152).

102. The three amplification steps described above in the Modular Approach line up with the three amplification steps of independent claims 1 and 14 of the '831 Patent as follows:

'831 claim step	Corresponding Fluidigm Step
1st amplification step	<i>"Pre-preamplification"</i> of multiple targets in a sample using target specific primers
2nd amplification step	<i>"Encoding preamplification"</i> of Step 1 products using tagged primers
3rd amplification step	<i>"Amplification"</i> of Step 2 products using tag-specific primers

Rationale (Motivation) Supporting Obviousness

103. As mentioned above, Fluidigm's disclosure is close to anticipating the claims. Fluidigm discloses using cell-free fetal DNA from a maternal blood sample in the three-step selective enrichment of claims 1 and 14, using more than 100 target polynucleotide sequences (in fact, 9,216) sequences. To the extent the standard for anticipation would not be met, the differences were minor and would be obvious.

104. One possible difference rests in the fact that the description of the maternal blood samples, aneuploidy detection, and the use of the library for sequencing are not found directly within the paragraphs that nominally relate to the Modular Approach (¶¶0115-0121) but are found elsewhere in Fluidigm. I will discuss these specific provisions that are found elsewhere below in more detail. However, at a high level, it is clear from the Fluidigm disclosure that there are certain statements in Fluidigm concerning blood samples, aneuploidy detection, and the use of the library for sequencing (e.g.) that are broadly applicable to each of the disclosed methods, and specifically applicable to the Modular Approach.

105. Independent claims 1 and 14 of the '831 Patent also require using "sequences selected from a chromosome tested for being aneuploid." Fluidigm expressly suggests the selection of sequences from a chromosome tested for being aneuploidy. For example, Fluidigm expressly teaches that the detection of target

sequence, which obviously need to be selected, are applicable to identifying the presence of aneuploidy:

“The methods of the invention are applicable to any technique aimed at detecting the presence or amount of one or more target nucleic acids in a nucleic acid sample. Thus, for example, these methods are applicable to identifying the presence of particular polymorphisms (such as SNPs), alleles, or haplotypes, or chromosomal abnormalities, such as amplifications, deletions, or aneuploidy.”

(Ex. 1003, ¶0175)(Emphases added)(Ex. 1004, ¶0150). Aneuploidy detection was also being conducted regularly in the course of pre-natal care in the relevant time frame. Because aneuploidy detection is the detection of an abnormal number of chromosomes (e.g., three copies of chromosome 21 when testing for Down syndrome), it would have been obvious to target sequences from a specific chromosome for which aneuploidy was suspected.

106. Also, the last part of independent claim 1 (but not claim 14) requires:

“wherein at least one primer of at least one of the second and third sets of primers includes a sequence configured to be added to the different non-random polynucleotide sequences to permit the enriched non-random polynucleotide sequences of the library to anneal to a same sequencing primer...”

(Ex. 1001, 63:66-64:42). Fluidigm teaches that its embodiments used for encoding

(including the Modular Embodiment) can introduce adaptor sequences. (Ex. 1003, ¶¶0160-161)(Ex. 1004, ¶¶0135-0136). Certain kinds of adaptor sequences were well-known to be used with NGS sequencing systems of the time. For example, Fluidigm teaches substituting methods for tagging nucleotide sequences for ligation, which was normally used to add sequencing adaptors. (Ex. 1003, ¶¶0160)(Ex. 1004, ¶¶0135). Sequencing adaptors are adaptors to which sequencing primers are used, for example, in the Solexa Genome Analyzer system. Note that the company Solexa was acquired by Illumina, and its Genome Analyzer later became the Illumina Genome Analyzer. The use of Solexa sequencing is expressly taught by Fluidigm. (Ex. 1003, ¶¶0161)(Ex. 1004, ¶¶0136). Based on Fluidigm's suggestion to use Solexa sequencing, and to substitute tagging methods for ligation to add adaptor sequences, it would have been obvious for one of ordinary skill in the art to have used one of the PCR-based amplification steps in Fluidigm's Modular Approach to add sequencing adaptors that included a same sequencing primer to the library molecules. The various amplification steps of Fluidigm's Modular Approach can be used to introduce an arbitrary tag sequence (within reasonable limits), and it would have been obvious to include a sequencing adaptor that included a same sequencing primer next to the tag sequences.

107. I will further explain the motivation supporting obviousness in the discussion relating to specific claim elements, below.

Graham Factors

108. I discuss the so-called *Graham* factors (referred to in ¶84, above) in the next few paragraphs, ¶¶109-112.

109. As discussed above in ¶90, the level of ordinary skill was high. The ordinary artisan in the relevant timeframe would have had a Ph.D. in molecular biology or a related field and approximately five years of experience working with or researching genetic testing, DNA amplification, and DNA sequencing.

110. The scope and content of the prior art are discussed throughout my declaration concerning each of the grounds.

111. The differences between the prior art and the claims are discussed in the “Overview of the Combination” and in the claim mapping, below.

112. I am not aware of any secondary considerations that would make an inference of non-obviousness more likely.

Reasonable Expectation of Success

113. It is my opinion that one of skill in the art in the relevant timeframe would have had a reasonable expectation of success in applying Fluidigm in a manner that meets the challenged claims of the '831 Patent.

114. As discussed above, by the relevant timeframe, the PCR method, upon which the three amplification steps of Fluidigm’s Modular Approach are described, was well-understood and predictable. In particular, one of ordinary skill in the art

would have been able to use the PCR reaction to target particular DNA sequences and would have been able to create primer pairs configured to add arbitrary nucleotide sequences (like “tags” and “adaptors”) to such targeted sequences. I note that the ’831 Patent itself does not provide anything new with respect to PCR amplification or the actual means by which one would introduce nucleotide tags or sequencing adaptors but is rather directed to the general idea of using PCR amplification in three steps while introducing tags and adaptors.

115. One of ordinary skill would have reasonably expected success in using Fluidigm’s Modular Approach to test samples for aneuploidy of a particular chromosome. As discussed above, in the relevant timeframe, next-generation sequencing was in widespread use. In fact, 454 Life Sciences Corporation’s sequencer, known as Genome Sequencer 20, was commercially in use by 2005. Likewise, Solexa’s next generation sequencer was released in late 2006 and achieved widespread commercial use by 2007. Barcoding or sample tagging was equally prevalent. Further, a kit for making a sequencing library for use with Solexa’s sequencer was commercially available in 2008, underscoring the routine nature of sequencing library preparation. Thus, one of ordinary skill would have expected success in being able to sequence cell-free DNA found in maternal blood.

116. It was further well-known by 2008 that one could isolate, amplify and sequence cell-free genomic DNA from maternal blood to detect aneuploidy,

including fetal trisomy 21. (Ex. 1006, Abstract)(Ex. 1013, Abstract)(Ex. 1003, ¶00175)(Ex. 1005, 25:10-20). As such, one of ordinary skill would have had a reasonable expectation of success in using cell-free DNA from maternal blood in a multi-step PCR method (such as Fluidigm's). One of ordinary skill would have expected reasonable success in identifying sequences from particular chromosomes, because the human genome had already been sequenced by the relevant timeframe. (Ex. 1057)("IHGSC"). Cell-free genomic DNA from maternal blood had also previously been successfully amplified and sequenced to detect aneuploidy, including fetal trisomy 21, trisomy 18 and trisomy 13. (Ex. 1006, Abstract)(Ex. 1013, Abstract). This typically involved amplifying DNA from a suspected chromosome and a reference chromosome and detecting whether levels of sequences of the suspect chromosome were elevated. (Ex. 1013, p. 001, right column). Given the success in using cell-free DNA to perform amplification and sequencing, one of ordinary skill would have had a reasonable expectation of success to employ cell-free DNA from maternal blood in the Fluidigm method with the goal of testing for chromosomal aneuploidy.

Fluidigm is Analogous Art

117. It is my opinion that Fluidigm and the other prior art cited in this Ground would be considered "analogous art" because it is in the same field as the '831 Patent (Fluidigm concerns DNA sequencing library preparation), and/or the

subject matter of the prior art would have been reasonably pertinent to the problems facing the named inventors.

Claim Mapping

118. This section maps the challenged claims to the relevant disclosures of Fluidigm, where the claim text appears in bold-italics, and the relevant mapping follows the claim text.

Claim 1

“1. A method for preparing a sequencing library from a maternal blood sample, the method comprising:”

119. Fluidigm states that its methods beginning with a sample. (Ex. 1003, ¶0116)(Ex. 1004, ¶0091). Fluidigm states that DNA “samples” of fetal DNA can come from **maternal blood**:

“Sample Preparation [¶] DNA or RNA useful in the methods described herein can be extracted and/ or amplified from any source.... For example, **samples of fetal DNA can be obtained from** an embryo (e.g., from one or a few embryonic or fetal cells) or from **maternal blood.**”

(Ex. 1003, ¶0122)(Emphases added)(Ex. 1004, ¶0097). Paragraph 0122 of Fluidigm obviously applies to the “Modular Approach,” because ¶0122 applies to “the methods described herein” which should be read as meaning “in the Fluidigm disclosure).” Furthermore, it makes technical sense that the Modular Approach would begin with such a sample.

120. Fluidigm describes using the methods to prepare a sequencing library, as in ¶¶0160-0161. For example, Fluidigm discloses that:

“If desired, tagged target nucleotide sequences generated as described herein may be analyzed by DNA sequencing.

Many current DNA sequencing techniques rely on ‘sequencing by synthesis.’ These techniques entail **library creation**, massively parallel PCR amplification of library molecules, and sequencing. Library creation starts with conversion of sample nucleic acids to appropriately sized fragments, ligation of adaptor sequences onto the ends of the fragments, and selection for molecules properly appended with adaptors.”

(Ex. 1003, ¶0160 see also ¶0177)(Emphases added)(Ex. 1004, ¶¶0135, 0152). As shown in the quote above, Fluidigm suggests analyzing the “nucleotide sequences generated as described herein” using DNA sequencing. Because the “Modular Approach” is “described herein” (*i.e.* in the Fluidigm disclosure) and because the Modular Approach is appropriate for producing a sequencing library, one of ordinary skill would have found it obvious that ¶0160 to applies to the Modular Approach.

121. Thus, one of skill in the art in the relevant timeframe, reading Fluidigm, would have found it obvious that Fluidigm’s Modular Approach was useful for preparing a sequencing library from a maternal blood sample.

“a. obtaining a maternal blood sample comprising fetal and maternal cell-free DNA;”

122. *See* immediately above. Fluidigm in ¶0122 describes using “samples of fetal DNA...from maternal blood.” (Ex. 1003, ¶0122)(Ex. 1004, ¶¶0097). This fetal DNA would have been understood in the relevant timeframe to include cell-free fetal and maternal DNA, and certainly cell-free fetal DNA was a focus at that time. (Ex. 1006, Abstract)(Ex. 1013, Abstract)(Ex. 1005, 5:29-6:8). For example, Fluidigm distinguishes between DNA obtained from cells and DNA obtained from bodily fluids, such as blood:

“Nucleic acids can be extracted or amplified **from cells, bodily fluids (e.g., blood, a blood fraction, urine, etc.),** or tissue samples by any of a variety of standard techniques.”

(Ex. 1003, ¶0122)(Emphasis added)(Ex. 1004, ¶0097). Fluidigm also distinguishes between fetal cells and fetal DNA in maternal blood:

“samples of fetal DNA can be obtained from an embryo (e.g., from one or a few embryonic or **fetal cells) or from maternal blood.**”

(Ex. 1003, ¶0122)(Emphasis added)(Ex. 1004, ¶0097). Fluidigm further indicates that a sample can simply be blood plasma, which is cell-free. (Ex. 1003, ¶0122)(Ex. 1004, ¶0097)(Ex. 1006, p. 001)(Ex. 1013, p. 001). Lo 2007A taught that maternal blood contains cell-free or extracellular fetal nucleic acids: “Since 1997, the progress of this field has been accelerated by **the unexpected finding of extracellular fetal nucleic acids in maternal plasma.**” (Ex. 1040, Abstract)(Emphasis added). These

disclosures would have indicated to one of ordinary skill that Fluidigm considered “blood” and “a blood fractions” to be a source separate from “cells,” (i.e., extracellular) and thus that Fluidigm contemplated using “cell-free” fetal and maternal DNA.

“b. selectively enriching a plurality of non-random polynucleotide sequences of genomic DNA from said fetal and maternal cell-free DNA to generate a library of enriched non-random polynucleotide sequences,”

123. As described above beginning in ¶¶94-106, Fluidigm teaches a process of selectively enriching of a plurality of non-random polynucleotide sequences of genomic DNA from said fetal and maternal cell-free DNA, to generate a library of those sequences. The Modular Approach of Fluidigm uses three targeted amplification steps, which are described above and in each of the three steps (i) – (iii), below.

124. The three steps (detailed below) lead to “selective enriching” because the PCR primers described in the Fluidigm Modular Approach anneal to, and thus cause amplification only of, sequences that contain complementary base pairs.

125. The “plurality of non-random polynucleotide sequences” required in claim 1 of the ’831 Patent are the sequences to which the primers anneal. These sequences are non-random, because they are effectively selected by the primers used, which Fluidigm describes as “target-specific primers.” (Ex. 1003, ¶0119)(Ex.

1004, ¶0094). There are 9,216 pairs of “target-specific primers” in the pre-amplification step of the Modular Approach, resulting in a plurality (e.g., 9,216) of targeted sequences. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094)

126. Fluidigm further teaches that the targeted sequences can be of genomic DNA. Regarding the targeted nucleic acids (polynucleotide sequences), Fluidigm states:

“Target Nucleic Acids [¶0124] **Any target nucleic acid** that can be tagged in an encoding reaction of the invention (described herein) can be detected using the methods of the invention....[¶0125] The targets can include, for example, nucleic acids associated with pathogens, such as viruses, bacteria, protozoa, or fungi; RNAs, e.g., those for which over- or under-expression is indicative of disease, those that are expressed in a tissue- or developmental-specific manner; or those that are induced by particular stimuli; **genomic DNA, which can be analyzed for specific polymorphisms (such as SNPs), alleles, or haplotypes, e.g., in genotyping. Of particular interest are genomic DNAs that are altered (e.g., amplified, deleted, and/or mutated) in genetic diseases or other pathologies; sequences that are associated with desirable or undesirable traits; and/or sequences that uniquely identify an individual (e.g., in forensic or paternity determinations).**”

(Ex. 1003, ¶¶0124-0125)(Emphases added)(Ex. 1004, ¶0094-0095). One of skill in

the art would have found it obvious that the disclosures in ¶¶0124 and 0125 of Fluidigm applies to the Modular Approach, because ¶0124 expressly relates the disclosure to an “encoding reaction of the invention (described herein),” meaning an invention described in Fluidigm. Paragraph 0124 states that sequences that “can be tagged in an encoding reaction” can be used. An encoding reaction (i.e., the “encoding preamplification”) is used in the Fluidigm Modular Approach, as described above in ¶99, and below under claim element (ii). This likewise indicates that ¶¶0124-0125 of Fluidigm are applicable to the Modular Approach. Finally, it was well-known in the relevant timeframe that fetal and maternal DNA from maternal blood included cell-free genomic DNA.

127. Fluidigm also discloses that the Modular Approach can be used to generate a library of enriched non-random polynucleotide sequences. The formation of a sequencing library is discussed above in ¶¶120-121, under the mapping preamble of claim 1. In short, Fluidigm states in ¶0160 that the methods “described herein” (*i.e.* described in Fluidigm) can be used to prepare a library for later sequencing. (Ex. 1003, ¶0160)(Ex. 1004, ¶0135). As further discussed immediately above, the library is a library of polynucleotide sequences that are enriched and non-random.

“wherein said plurality of non-random polynucleotide sequences comprises at least 100 different non-random polynucleotide sequences selected from a chromosome tested for being aneuploid, said enriching comprising:”

128. The Modular Approach of Fluidigm ultimately results in 9,216 different non-random polynucleotide sequences in the library, which is at least 100 different sequences. (Ex. 1003, ¶¶0119 and ¶0116)(Ex. 1004, ¶0094).

129. Fluidigm makes clear that the sequences are both different and non-random, by stating that “target specific primers for 9216 different target nucleic acids can be employed in one mixture.” (Ex. 1003, ¶¶0119)(Ex. 1004, ¶0094). The “nucleic acid” is cell-free genomic DNA, and a polynucleotide sequence. (Ex. 1003, ¶¶0042-0043)(Ex. 1004, ¶0094). Note that Fluidigm states that “[t]he term ‘target nucleic acids’ is used herein to refer to particular nucleic acids to be detected in the methods of the invention.” (Ex. 1003, ¶0048)(Emphasis added)(Ex. 1004, ¶0034). Thus, the use of the term “target-specific primers” indicates that the sequences are non-random. The same 9,216 sequences are retained throughout the entire Modular Approach.

130. Fluidigm also teaches that the methods can be used to create a library for aneuploidy detection. Fluidigm discloses that:

“The methods of the invention are applicable to any technique aimed at **detecting the** presence or **amount of one or more target nucleic acids** in a nucleic acid sample. Thus, for

example, **these methods are applicable to identifying the presence of** particular polymorphisms (such as SNPs), alleles, or haplotypes, or **chromosomal abnormalities**, such as amplifications, deletions, or **aneuploidy.**”

(Ex. 1003, ¶¶0175)(Emphases added)(Ex. 1004, ¶¶0150). One of ordinary skill would have found it obvious to use the Modular Approach of Fluidigm to test for aneuploidy, because of the express suggestion quoted above. The suggestion applies to “the methods of the invention”, *i.e.* the methods in Fluidigm, including the Modular Approach.

131. It would have been obvious based on Fluidigm to test sequences relating to a single chromosome. Fluidigm expressly suggests testing for aneuploidy, which is an abnormal number of chromosome(s), or parts of a chromosome. For example, an extra copy of chromosome 21 resulted in the condition trisomy 21 (also known as “Down syndrome”). When testing for aneuploidy (as expressly suggested by Fluidigm), it would have been obvious to target sequences on the chromosome suspected of aneuploidy to determine. This would have allowed one of ordinary skill to count the sequences present, to determine if there was an elevated level of sequences from the chromosome suspected of trisomy. Fluidigm expressly teaches, for example, that “the presence or **amount** of one or more target nucleic acids” can lead to a detection of aneuploidy. (Ex. 1003, ¶¶0175)(Emphasis added)(Ex. 1004, ¶¶0150). The detection of the

amount of a target nucleic acid on a chromosome (as compared to a reference, e.g. a chromosome not being tested for aneuploidy) would obviously have been a way to test for aneuploidy, which is an abnormal number of a particular chromosome. For example, it would have been obvious that an extra copy of chromosome 21 in the fetal genome would have led to elevated levels of sequences from chromosome 21. It would further have been obvious that using more sequences on chromosome 21 would have been more likely to produce a confident diagnosis.

132. Such counting was a well-known technique in the relevant timeframe. For example, Chiu taught detecting trisomy 21 (“T21”) by testing a large number of sequences on chromosome 21 (“chr21”) and looking for elevated levels of chromosome 21 sequences in maternal blood. Chiu explains:

“[F]etal T21 is diagnosed by **detecting the small increment in the total amount of the chr21 gene locus** contributed by the trisomic chr21 in the fetus as **compared with a gene locus on a reference chromosome**. The proportional increment in chr21 sequences is expectedly small because fetal DNA contributes only a minor fraction of DNA in **maternal plasma** (8). To reliably detect the small increase, **a large absolute number of chr21 and reference chromosome sequences** of the loci targeted by the digital PCR assays **need to be analyzed....**”

(Ex. 1013, p. 001, right column)(Emphases added).

133. Likewise, Fan 2008 taught that “[b]y counting the number of sequence tags mapped to each chromosome, the over- or underrepresentation of any chromosome in maternal plasma DNA contributed by an aneuploid fetus can be detected.” (Ex. 1006, p. 001, right column).

134. Moreover, targeting larger numbers of sequences on the same chromosome would obviously have increased the chance of detecting an abnormal number of copies of a chromosome in a statistically reliable way (e.g., based on the use of large numbers). For example, Chiu disclosed that:

“To reliably detect the small increase, a large absolute number of chr21 and reference chromosome sequences of the loci targeted by the digital PCR assays need to be analyzed...”

(Ex. 1013, p. 001, right column). One of ordinary skill would have understood this to mean that by testing additional sequences on the same chromosome, the ability to more reliably detect aneuploidy would have been increased.

“(i) a first amplification step to generate a plurality of first reaction products, said amplification comprising at least 100 first primers configured to amplify at least 100 different non-random polynucleotide sequences;”

135. As discussed above in ¶98, the claimed first amplification step in the Modular Approach is the optional “pre-pre-amplification” step. In the pre-pre-amplification step, the sample is selectively enriched by PCR amplification, using

9,216 first primer pairs. Fluidigm discloses:

“To increase target nucleic acid concentration prior to encoding, an optional pre-preamplification reaction can be carried out before the encoding preamplification reaction. The pre-preamplification can be carried out in multiplex [sic: multiplex]. For example, **target-specific primers for 9216 different target nucleic acids** can be employed in one mixture.”

(Ex. 1003, ¶119)(Emphasis added)(Ex. 1004, ¶10094)

136. The pre-pre-amplification step “increase[s] target nucleic acid concentration.” (Ex. 1003, ¶119)(Ex. 1004, ¶10094), and thus provides a plurality of first reaction products, in the form of copies of the target sequences.

137. The pre-pre-amplification amplifies 9,216 “different target nucleic acids.” (Ex. 1003, ¶119)(Ex. 1004, ¶10094). As explained directly above, “different target nucleic acids” means that the nucleic acids (polynucleotide sequences) are both different and non-random.

“(ii) a second amplification step to generate a second reaction product, said amplification comprising a second set of primers comprising sequences contained in the first reaction products;”

138. As discussed above in ¶¶99-100, the Fluidigm Modular Approach uses a second amplification step with a second set of primers. The Modular Approach performs an “encoding pre-amplification” step. (Ex. 1003, ¶119)(Ex. 1004,

¶0094). The “encoding pre-amplification” step divides the enriched sample produced by the pre-pre-amplification step into a series of different aliquots. In this particular case, the number chosen is 96 aliquots. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The 96 aliquots then each undergo a PCR amplification using, for example, 96 primer pairs in each aliquot. (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). The 96 primer pairs per aliquot can contain “nucleotide tag pairs.” (Ex. 1003, ¶0119)(Ex. 1004, ¶0094). These nucleotide tag pairs (also known as “bar codes” or “encoding sequences”) are sequences whose purpose is to identify samples from which the DNA sequences were taken (for example). (Ex. 1003, ¶¶0005, 0064, 0094, 0115)(Ex. 1004, ¶¶0005-0008, 0071, 0077-0078, 0082, 0090). Fluidigm describes the “encoding pre-amplification” step immediately after describing the “pre-pre-amplification step,” as follows:

“The pre-preamplification can be carried out in multiplex [sic: multiplex]. For example, target-specific primers for 9216 different target nucleic acids can be employed in one mixture. **This mixture can then be divided into R=96 aliquots and each aliquot subjected to an encoding preamplification reaction** on a microfluidic device, using **T=96 different primer pairs** that add 96 different nucleotide tag pairs to the target nucleotide sequences in each of the 96 aliquots.”

(Ex. 1003, ¶0119)(Emphases added)(Ex. 1004, ¶0094).

139. The “encoding preamplification” step uses a second set of primers,

where the second set of primers comprises sequences contained in the first reaction products. This is because the pre-pre-amplification step amplifies the target sequences for the entire Modular Approach. The encoding amplification step then targets these same sequences, which have been divided into 96 aliquots. Fluidigm discloses that:

“To increase target nucleic acid concentration prior to encoding, an optional pre-preamplification reaction can be carried out before the encoding preamplification reaction. The pre-preamplification can be carried out in multiplex. For example, **target-specific primers for 9216 different target nucleic acids** can be employed in one mixture. This mixture can then be divided into R=96 aliquots and each aliquot subjected to an encoding preamplification reaction on a microfluidic device, using T=96 different primer pairs that add 96 different nucleotide tag pairs to **the target nucleotide sequences** in each of the 96 aliquots.”

(Ex. 1003, ¶0119)(Emphases added)(Ex. 1004, ¶0094). Because the same sequences are being targeted, portions of the primers in the second amplification step (the “encoding amplification”) must be complementary to at least portions of the target sequences that were the first reaction product. Because a primer sequence is complementary to one polynucleotide sequence in a DNA molecule, the primer must comprise at least a portion of the complementary sequence on the other strand of a DNA molecule. Thus, the second set of primers “compris[es] sequences contained

in the first reaction products.”

140. Furthermore, Fluidigm expressly states that “[t]o increase specificity, the primers employed for preamplification can be nested relative to primers employed for pre-preamplification.” (Ex. 1003, ¶¶0119)(Ex. 1004, ¶¶0094). Fluidigm discloses that:

“A second primer pair is ‘nested’ relative to a first primer pair if the first primer pair is employed to amplify a first amplification product and then the second primer pair is employed to amplify a target nucleotide sequence within the first amplification product. Nesting can be used increase the specificity of the amplification reaction.”

(Ex. 1003, ¶¶0058)(Emphasis added)(Ex. 1004, ¶¶0044). This further demonstrates that the second set of primers compris[es] sequences contained in the first reaction products.

141. The second amplification step produces a second reaction product, by amplifying the target nucleic acid sequences and adding encoding sequences to them. (Ex. 1003, ¶¶0119, 0116)(Ex. 1004, ¶¶0094).

“and (iii) a third amplification step to generate a third reaction product comprising said library of enriched non-random polynucleotide sequences, said amplification comprising a third set of primers comprising sequences contained in the second reaction products;”

142. As described above in ¶¶100-101, Fluidigm describes a third

amplification step that follows the “encoding preamplification” step. In the third (“amplification”) step, the (*e.g.* 96) separate aliquots containing tagged DNA can be added to separate chambers in a microfluidic device. (Ex. 1003, ¶0120)(Ex. 1004, ¶0095). From there, each aliquot will be *subdivided* 96 times, and each sub-aliquot is subjected to amplification using a single PCR primer pair specific to the sequence tag combinations employed in the “encoding preamplification.” (Ex. 1003, ¶0120)(Ex. 1004, ¶0095). Fluidigm discloses that:

“After the encoding preamplification reaction, amplification can be carried out in separate chambers of a microfluidic device. For example, each of the 96 aliquots produced upon encoding preamplification can be loaded into individual sample lines of a matrix-type micro fluidic device, and **each of 96 different tag-specific primer combinations can be loaded into individual assay columns.** Each different of the 96 primer combination can amplify a different target nucleic acid in each of the 96 aliquots. **The resulting 9216 reaction chambers (sub-aliquots) can then be subjected to amplification,** followed by detection of amplification product(s)....”

(Ex. 1003, ¶0120)(Emphases added)(Ex. 1004, ¶0095). As noted in the bolded language above, the primers used in the amplification step are “tag-specific primer combinations.” (Ex. 1003, ¶0120)(Ex. 1004, ¶0095). This means that the primers anneal to portions of the encoding tags that were included in the previous encoding

amplification step. To do so, these primers must be complementary to portions of the tag sequences that are the second amplification product. Because a primer is complementary to one polynucleotide sequence in a DNA molecule, the primer must be comprised at least a portion of the complementary sequence on the other strand of a DNA molecule. Thus, the third set of primers “comprising sequences contained in the second reaction products.”

143. The output of the amplification step is 9,216 aliquots, each with a specific, enriched target (*i.e.* non-random) sequence. This is the third amplification product comprising said library of enriched non-random polynucleotide sequences.

“wherein at least one primer of at least one of the second and third sets of primers includes a sequence configured to be added to the different non-random polynucleotide sequences to permit the enriched non-random polynucleotide sequences of the library to anneal to a same sequencing primer for the enriched non-random polynucleotide sequences of the library.”

144. Fluidigm also renders obvious using the primers of the second or third amplification step to add a sequence that anneals to a sequencing primer. Fluidigm describes that the various library preparation methods it discloses can be used to prepare sequencing libraries to be used in later sequencing. (Ex. 1003, ¶¶0160-161, 0177)(Ex. 1004, ¶¶0135-0136, 0152). To facilitate the use in later sequencing, Fluidigm teaches adding adaptors through the tagging amplification steps. Fluidigm states that:

“If desired, tagged target nucleotide sequences generated as described herein may be analyzed by DNA sequencing. Many current DNA sequencing techniques rely on ‘sequencing by synthesis.’ These techniques entail library creation, massively parallel PCR amplification of library molecules, and sequencing. Library creation starts with conversion of sample nucleic acids to appropriately sized fragments, ligation of adaptor sequences onto the ends of the fragments, and selection for molecules properly appended with adaptors. The presence of the adaptor sequences on the ends of the library molecules enables amplification of random- sequence inserts. The above-described methods for tagging nucleotide sequences can be substituted for ligation, to introduce adaptor sequences.”

(Ex. 1003, ¶0160)(Emphases added)(Ex. 1004, ¶0135).

145. Fluidigm also teaches that “Solexa” sequencing as an example of sequencing that can be used with the Fluidigm methods:

“In particular embodiments, the number of library DNA molecules produced in the massively parallel PCR step is low enough that the chance of two molecules associating with the same substrate, e.g. the same bead (in 454 DNA sequencing) or the same surface patch (in **Solexa** DNA sequencing) is low, but high enough so that the yield of amplified sequences is sufficient to provide a high throughput in automated sequencing.”

(Ex. 1003, ¶0161)(Emphasis added)(Ex. 1004, ¶0136). This is relevant because Solexa sequencing in the relevant timeframe was well-known to use a “**same sequencing primer**” (called a “universal primer for sequencing” in Bentley). (Ex. 1008, p. 001). In Solexa sequencing, two sequencing adaptors are added to library molecules, which are then amplified by bridge PCR. The bound templates from the library are made single stranded. A single same or universal sequencing primer is then annealed to each template having respective sequencing adaptor. This approach has the advantage of only requiring one single, same sequencing primer for each library molecule. (Ex. 1008, p. 001).

146. It would have been obvious to use the primers in the amplification (second or third) steps of Fluidigm to introduce sequencing adaptors into the library sequences of Fluidigm’s Modular approach. This is because Fluidigm states that its methods can be used for sequencing analysis, and expressly states that Solexa sequencing can be used. In Solexa sequencing, it is necessary to introduce an adaptor to the library sequences prior to sequencing. When sequencing occurs, a same sequencing primer anneals to the adaptor. (Ex. 1008, p. 001).

147. Fluidigm also suggests that the adaptors otherwise introduced by ligation could be added using the tagging nucleotide sequences. (Ex. 1003, ¶0160)(Ex. 1004, ¶0135). In Solexa sequencing, the sequencing adaptors were typically introduced by ligation. (Ex. 1008, p. 001)(Ex. 1012, Fig. 1). As noted

above, Fluidigm teaches substituting its encoding methods (including the Modular Approach) for such ligation, specifically to add adaptors. (Ex. 1003, ¶0160)(Ex. 1004, ¶0135). It would have been obvious that the adaptors could be introduced by using PCR primers that contain one or more adaptor sequences. It was well-known in the relevant timeframe that PCR primers could be used to introduce arbitrary sequences of reasonable length. For example, Fluidigm teaches using PCR primers in the “encoding amplification” step to introduce DNA sequence tags (Ex. 1003, ¶0119)(Ex. 1004, ¶0094), which are arbitrary sequences used simply to identify different samples. (Ex. 1003, ¶¶0101, 0064, 0094)(Ex. 1004, ¶¶0080, 0050, 0082). This conclusion is reinforced by the fact that Fluidigm teaches that encoding PCR and encoding ligation can both be used to add a tag sequence. In Fluidigm’s words:

“Nucleotide tags can be added, for example, by an ‘**encoding PCR**’ in which the at least **one primer comprises a target-specific portion and a nucleotide tag** located on the 5’ end of the target-specific portion, and **a second primer that comprises only a target-specific portion or a target-specific portion and a nucleotide tag** located on the 5’ end of the target-specific portion. For illustrative examples of PCR protocols applicable to encoding PCR, see pending WO Application US03/37808 as well as U.S. Pat. No. 6,605,451. **Nucleotide tags can also be added by an ‘encoding ligation’ reaction** that can comprise a ligation reaction in which at least one primer comprises a target-specific portion and nucleotide

tag located on the 5' end of the target-specific portion, and a second primer that comprises a target-specific portion only or a target-specific portion and a nucleotide tag located on the 5' end of the target specific portion.”

(Ex. 1003, ¶¶0062)(Emphases added)(Ex. 1004, ¶¶0048).

148. Fluidigm also teaches that tag sequences introduced by PCR can be functional. For example, Fluidigm teaches that tag sequences can anneal to a later-employed PCR primer. (Ex. 1003, ¶¶0086, 0092, 0108, 0119)(Ex. 1004, ¶¶0074, 0085, 0094). Because one can introduce arbitrary functional sequences using PCR primers (known to be an alternative to ligation), it would have been obvious to introduce a sequencing adaptor sequence to perform Solexa sequencing, as expressly suggested by Fluidigm.

149. Furthermore, it would have been obvious to introduce sequencing adaptors to include a same sequencing primer in the third (“amplification step”) because the primers used in this step can anneal to the encoding tags introduced in the “encoding pre-amplification step,” thus requiring fewer primers overall.

150. In this way it would have been obvious to have at least one primer of at least one of the second and third sets of primers (*i.e.* the primers of the [third] amplification step of Fluidigm’s Modular Approach) to include a sequence (*i.e.* a sequencing adaptor for Solexa sequencing) configured to be added to the different non-random polynucleotide sequences (because the adaptors will be added in the

Modular Approach's [third] amplification step) to permit the enriched non-random polynucleotide sequences of the library to anneal to a same sequencing primer (*i.e.* the Solexa sequencing primer) for the enriched non-random polynucleotide sequences of the library (*i.e.* the output of Fluidigm's Modular Approach).

Claim 2

“2. The method of claim 1, wherein at least one primer of at least one of the second and third sets of primers includes a sequence configured to be added to the different non-random polynucleotide sequences to add an index to the enriched non-random polynucleotide sequences of the library, the index being indicative of the maternal blood sample from which the library was generated.”

151. In my opinion, claim 2 of the '831 Patent would also have been obvious in view of Fluidigm. For example, Fluidigm teaches that samples can be mixed, and when mixed, can use sample-specific nucleotide tags to identify the origin of the sample (*e.g.*, the person from whom the sample was taken). (Ex. 1003, ¶¶0002, 0005, 0062, 0094, 0115)(Ex. 1004, ¶¶0005-0008, 0071, 0077-0078, 0082).

152. Specifically, Fluidigm teaches an “encoding amplification” step that adds a nucleotide tag or index using a second set of primers, as discussed above in ¶¶99 and 138-141. This constitutes at least one primer of at least one of the second set of primers including a nucleotide tag or index sequence configured to be added to the different non-random polynucleotide sequences.

153. It would have been obvious to use a primer in the second step of Fluidigm's Modular Approach to add an index to the enriched non-random

polynucleotide sequences of the library, the index being indicative of the maternal blood sample from which the library was generated.

154. Fluidigm teaches that samples from different individuals can be mixed, and before they are mixed, the samples should be tagged to identify the origin of the sample:

“In certain embodiments, the method entails providing **S samples that will be mixed together** prior to assay, where S is an integer greater than 1. Each of these samples can be **separately subjected to an encoding reaction** that produces a set of T tagged target nucleotide sequences, **each tagged target nucleotide sequence comprising a sample specific nucleotide tag** and a target nucleotide sequence.”

(Ex. 1003, ¶0005)(Emphases added)(Ex. 1004, ¶0005).

155. Fluidigm also defines a “sample-specific nucleotide tag” as follows:

“The term ‘sample-specific’ nucleotide tag is used herein to refer to **a nucleotide tag that encodes the identity of the sample** of the target nucleotide sequence to which the tag is, or becomes, linked in an encoding reaction.”

(Ex. 1003, ¶0064)(Emphasis added)(Ex. 1004, ¶0050). In the context of the present Ground, the tag is an index that is indicative of the maternal blood sample from which the library was generated.

156. In my opinion, it would have been obvious to add a sample-specific

nucleotide tag using the second set of primers. These primers were already intended to add tags (*see* discussion in ¶¶138-141, above). Based on Fluidigm’s teaching to mix samples and track them using a sample-specific nucleotide tag, it would have been obvious to do so. One of ordinary skill would also have had a reasonable expectation of success, because (as discussed above in ¶147), it was well-known that one could add arbitrary sequences of reasonable length using PCR primers.

Claim 3

“3. The method of claim 1, wherein at least one primer of the third set of primers includes a sequence configured to be added to the different non-random polynucleotide sequences to add an index to the enriched non-random polynucleotide sequences of the library, the index being indicative of the maternal blood sample from which the library was generated.”

157. In my opinion, claim 3 of the ’831 Patent would also have been obvious in view of Fluidigm, for largely the same reasons discussed above under claim 2, ¶¶151-156. Fluidigm teaches mixing samples and using a sample-specific nucleotide tag to track the sample’s origin. (Ex. 1003, ¶0002, 0094)(Ex. 1004, 0082). The sample-specific nucleotide tags could be added by PCR, including during the third (amplification) step explained above in ¶¶142-143 and 100-101. It would have been obvious to one of ordinary skill in the relevant timeframe to add the sample-specific nucleotide tag prior to mixing samples. It would further have been apparent that this could happen during any of the three PCR-based amplification steps, including the third (“amplification”) step. Thus, it would have

been obvious to use at least one primer of the third set of primers having a sample-specific nucleotide sequence (*i.e.* a sequence configured to be added to the different non-random polynucleotide sequences to add an index to the enriched non-random polynucleotide sequences of the library). This would have had the added advantage of requiring fewer primers, because the primers could target tags added in the second step. (Ex. 1003, ¶¶0119)(Ex. 1004, ¶¶0094). This is because the sample-specific nucleotide sequence “encodes the identity of the sample” (Ex. 1003, ¶¶0064)(Ex. 1004, ¶¶0050), it is indicative of the maternal blood sample from which the library was generated.

Claim 4

“4. The method of claim 1, wherein primers of the second set of primers contain universal sequences such that nucleic acids of the second reaction product contain common universal sequences added to the different non-random polynucleotide sequences, and wherein the third set of primers are configured to hybridize at least in part to the common universal sequences.”

158. In my opinion, claim 4 of the '831 Patent would also have been obvious in view of Fluidigm. For example, Fluidigm teaches that each aliquot in the encoding amplification step is amplified using primers (*i.e.* primers of the second set of primers) that introduce tags (*i.e.* the common universal sequences) to which primers in the third set of primers (*i.e.* primers in the amplification step) are configured to hybridize. Fluidigm discloses that:

“Each of the R aliquots can be separately subjected to an

encoding reaction that produces a set of T tagged target nucleotide sequences, wherein T is the number of target nucleic acids to be detected in each aliquot, T being an integer greater than one (e.g., 96). Each tagged target nucleotide sequence includes a first nucleotide tag 5' of a target nucleotide sequence, a target nucleotide sequence, and a second nucleotide tag 3' of the target nucleotide sequence. The combination of nucleotide tags in each of said T tagged target nucleotide sequences is unique for every tagged target nucleotide sequence in each aliquot. However, **the same set of first and second nucleotide tag combinations is used in the encoding reaction in each of the aliquots.**"

(Ex. 1003, ¶0116)(Emphasis added)(Ex. 1004, ¶0091).

159. This approach allows Fluidigm to re-use tag sequences. As Fluidigm states that:

"Within each module, the sets of tag pairs differ from one another, but same set of tag pairs is used for each module."

(Ex. 1003, ¶0115)(Ex. 1004, ¶0090).

160. For this reason, tag combinations present in one aliquot are re-used in other aliquots:

"[I]n certain embodiments, the combination of nucleotide tags in each of said T tagged target nucleotide sequences is present in a tagged target nucleotide sequence in each of the other aliquots, although each tag combination can be attached to a

different target nucleotide sequence.”

(Ex. 1003, ¶0116)(Ex. 1004, ¶0091). Because the same tag-pair will be present in each of the 96 aliquots and attached to different target sequences, the various tag pairs are common universal sequences.

161. The third set of primers hybridizes to these sequences, because the third set of primers (in the “amplification step”) are tag-specific primers, as Fluidigm explains in ¶0120. (Ex. 1003, ¶0120)(“96 different **tag-specific primer combinations** can be loaded into individual assay columns”)(Emphasis added)(Ex. 1004, ¶0095). This means that the third set of primers are configured to hybridize to the tags introduced in the second step (the “encoding amplification”).

Claim 5

“5. The method of claim 4, wherein at least one primer of the third set of primers includes a sequence configured to be added to the different non-random polynucleotide sequences to add an index to the enriched non-random polynucleotide sequences of the library, the index being indicative of the maternal blood sample from which the library was generated.”

162. In my opinion, claim 5 of the ’831 Patent would also have been obvious in view of Fluidigm. Claim 5 is obvious over Fluidigm for the same reasons given for claim 3, as discussed in ¶157. The requirement of intervening claim 4 does not alter the analysis presented under claim 3.

Claim 6

“6. The method of claim 1, wherein said plurality of non-random polynucleotide sequences comprises at least 300 different non-random polynucleotide sequences selected from the chromosome tested for being aneuploid.”

163. In my opinion, claim 6 of the '831 Patent would also have been obvious in view of Fluidigm. As explained above in ¶¶131-134, it would have been obvious to test a specific chromosome for being aneuploid based on Fluidigm's suggestion to use the “amount or presence” of target sequences to test for aneuploidy, and the known successes in performing similar analyses. (Ex. 1003, ¶0175)(Ex. 1004, ¶0150)(Ex. 1006, p. 001, right column)(Ex. 1013, p. 001, right column). Because Fluidigm's modular approach uses 9,216 sequences, it would have been obvious to have at least 300 such sequences come from the chromosome being tested for aneuploidy, in order to determine whether the amount of such sequences was abnormally high, for example in the case of Down syndrome. It would have been obvious that testing more sequences would result in a higher chance to correctly detect aneuploidy. Chromosomes are typically very long in length compared to those polynucleotide templates that are sequenced with next generation sequencers. Targeting larger numbers of sequences on a chromosome would obviously have increased the chance of detecting an abnormal number of copies of a chromosome in a statistically reliable way. For example, Chiu in 2008 stated that:

“To reliably detect the small increase, a large absolute number of chr21 and reference chromosome sequences of the loci

targeted by the digital PCR assays need to be analyzed....”

(Ex. 1013, p. 001, right column). One of ordinary skill would have understood this to mean that by testing additional sequences on the same chromosome, the ability to more reliably detect aneuploidy would have been increased.

Claim 7

“7. The method of claim 1, wherein said plurality of non-random polynucleotide sequences comprises at least 500 different non-random polynucleotide sequences selected from the chromosome tested for being aneuploid.”

164. In my opinion, claim 7 of the ’831 Patent would also have been obvious in view of Fluidigm. For the same reasons explained above for claim 6 in ¶162, it would have been obvious to have at least 500 of such sequences from the chromosome being tested for being aneuploidy.

Claim 10

“10. The method of claim 1, wherein said selectively enriching comprises performing PCR.”

165. In my opinion, claim 10 of the ’831 Patent would also have been obvious in view of Fluidigm. The “selectively enriching” of claim 1 comprises steps b(i), (ii) and (iii). Each of these steps use primers, which indicates the use of PCR. (Ex. 1003, ¶¶0119-0120)(Ex. 1004, ¶0094-0095).

166. Fluidigm also teaches that the “encoding amplification” step (the second amplification) of the Modular Approach can use PCR. Fluidigm states that:

“As used herein, the term ‘**encoding reaction**’ refers to reaction in which at least one nucleotide tag is added to a target nucleotide sequence. Nucleotide tags can be added, for example, by an ‘**encoding PCR**’ in which the at least one primer comprises a target-specific portion and a nucleotide tag located on the 5’ end of the target-specific portion, and a second primer that comprises only a target-specific portion or a target-specific portion and a nucleotide tag located on the 5’ end of the target-specific portion.”

(Ex. 1003, ¶¶0062)(Ex. 1004, ¶¶0048).

Claim 14

167. In my opinion, claim 14 of the ’831 Patent would also have been obvious in view of Fluidigm.

“14. A method for preparing a sequencing library from a maternal blood sample, the method comprising:”

168. The preamble of claim 14 of the ’831 Patent is disclosed in the same manner as the preamble of claim 1, as discussed in ¶¶119-121.

“a. obtaining a maternal blood sample comprising fetal and maternal cell-free DNA;”

169. Element a. of claim 14 of the ’831 Patent is disclosed in the same manner as Element a. of claim 1, as discussed in ¶122

“b. selectively enriching a plurality of non-random polynucleotide sequences of genomic DNA from said fetal and maternal cell-free DNA to generate a library of enriched non-random polynucleotide sequences,”

170. Element b. of claim 14 of the '831 Patent is disclosed in the same manner as Element b. of claim 1, as discussed in ¶¶124-134.

“wherein said plurality of non-random polynucleotide sequences comprises at least 100 different non-random polynucleotide sequences selected from a chromosome tested for being aneuploid, said enriching comprising:”

171. Element b. of claim 14 of the '831 Patent is disclosed in the same manner as Element b. of claim 1, as discussed in ¶¶124-134.

“(i) amplifying said plurality of non-random polynucleotide sequences from said maternal and fetal genomic DNA using a first pair of primers,”

172. Element b(i). of claim 14 of the '831 Patent is disclosed in the same manner as Element b(i). of claim 1, as discussed in ¶¶135-137.

“wherein said plurality of non-random polynucleotide sequences comprises at least 100 different non-random polynucleotide sequences selected from a chromosome tested for being aneuploid;”

173. Element b(i). of claim 14 of the '831 Patent is disclosed in the same manner as Element b(i). of claim 1, as discussed in ¶¶124-137.

“(ii) amplifying the product of (i) with a second set of primers;”

174. Element b(ii). of claim 14 of the '831 Patent is disclosed in the same manner as Element b(ii). of claim 1, as discussed in ¶¶138-141, noting that this claim element is broader than the corresponding element of claim 1.

“(iii) amplifying the product of (ii) with a third set of primers;”

175. Element b(iii). of claim 14 of the '831 Patent is disclosed in the same manner as Element b(iii). of claim 1, as discussed in ¶¶142-143, noting that this claim element is broader than the corresponding element of claim 1.

“and wherein one of said second or third sets of primers includes an indexing sequence.”

176. This element of claim 14 of the '831 Patent is disclosed in the same manner as claim 2, as discussed in ¶¶151-156, noting that this claim element is broader than both claim 2 and distinct from the last “wherein” clause of claim 1.

Claim 15

“15. The method of claim 14, wherein said third set of primers includes the indexing sequence.”

177. In my opinion, claim 15 of the '831 Patent would also have been obvious in view of Fluidigm. Claim 15 is obvious over Fluidigm for the same reasons given for claim 3, as discussed in ¶157. The dependency from claim 14 does not alter the analysis presented under claim 3.

Claim 16

“16. The method of claim 14, wherein said indexing sequence distinguishes polynucleotides in the maternal blood sample from polynucleotides in a different sample.”

178. In my opinion, claim 16 of the '831 Patent would also have been obvious in view of Fluidigm. As discussed above under claim 2 (§§151-156), Fluidigm renders obvious using “sample-specific” DNA tags. (Ex. 1003, ¶0075)(Ex. 1004, ¶0061). Sample-specific tags “encode[] the identity of the sample of the target nucleotide sequence to which the tag is, or becomes, linked in an encoding reaction.” (Ex. 1003, ¶0064)(Ex. 1004, ¶0050). Because the tag “encodes the identity” of a sample, and is attached to particular sequence from that sample, the tag obviously distinguishes sequences in different samples. The tags are obviously different, because when samples are pooled in a multiplexed run (*see* ¶149, below), the tags must have distinct sequences in order to be able to separate distinguish sequence reads from different samples.

Claim 17

“17. The method of claim 14, wherein said cell-free DNA is from a plurality of different individuals, and wherein said indexing sequence identifies a maternal blood sample from each of said plurality of different individuals.”

179. In my opinion, claim 17 of the '831 Patent would also have been obvious in view of Fluidigm. It would specifically have been obvious to use cell-free DNA from a plurality of individuals, where each individual's sequences are

identified with an index sequence. Fluidigm expressly discloses that:

“In various embodiments, methods are provided for detecting a plurality of target nucleic acids (i.e., T target nucleic acids, where T is an integer greater than one) in a plurality of samples. In certain embodiments, the method entails providing **S samples that will be mixed together** (i.e., pooled) prior to assay, where S is an integer greater than 1. Each of these samples is separately subjected to an encoding reaction that produces a set of T tagged target nucleotide sequences, wherein **each nucleotide tag encodes information about the identity and/or sample source of a particular target nucleic acid**. For each of these S samples (i.e., each of the samples to be pooled), the tagged target nucleotide sequences are mixed to form an assay mixture.”

(Ex. 1004, ¶¶0005, 0086)(Emphases added)(Ex. 1004, ¶¶0005-0008, 0071, 0077-0078, 0082). In the Fluidigm Modular Approach, it would have been obvious to combine samples after the encoding amplification step described above (*i.e.* after adding sample-specific tags), because if added earlier one would not be able to use the tags to distinguish the samples.

Claim 18

“18. The method of claim 14, wherein said plurality of non-random polynucleotide sequences comprises at least 300 different non-random polynucleotide sequences selected from the chromosome tested for being aneuploid.”

180. In my opinion, claim 18 of the '831 Patent would also have been obvious in view of Fluidigm. For the same reasons explained above for claim 6 in ¶162, it would have been obvious to have at least 300 of such sequences from the chromosome being tested for being aneuploidy. The change in dependency to claim 14 does not affect the analysis.

Claim 19

“19. The method of claim 14, wherein said plurality of non-random polynucleotide sequences comprises at least 500 different non-random polynucleotide sequences selected from the chromosome tested for being aneuploid.”

181. In my opinion, claim 19 of the '831 Patent would also have been obvious in view of Fluidigm. For the same reasons explained above for claim 6 in ¶162, it would have been obvious to have at least 500 of such sequences from the chromosome being tested for being aneuploidy. The change in dependency to claim 14 does not affect the analysis.

Claim 22

“22. The method of claim 14, wherein said selectively enriching comprises performing PCR.”

182. In my opinion, claim 22 of the '831 Patent would also have been obvious in view of Fluidigm. For the same reasons explained above for claim 10 in ¶¶165-166, Fluidigm discloses and renders obvious the use of PCR, because each of its three amplification steps in the Modular Approach use primers, and the “encoding

amplification” step is expressly PCR.

Ground 2. Claims 1-10, 13-22 and 24 are obvious as in Ground 1 in further view of Sequenom.

183. It is further my opinion that claims 1-10, 13-22 and 24 of the ’831 Patent would have been obvious over Fluidigm and the knowledge of one of ordinary skill, as applied above in Ground 1, in view of WO2009/032781A2 (“Sequenom”)(Ex. 1005).

Overview of the Combination

184. In my analysis for Ground 2, I am using Fluidigm and the knowledge of one of ordinary skill in the same manner as discussed under Ground 1 (¶¶93-182), and the discussion from Ground 1 is applies to Ground 2 in the same way. Sequenom provides additional support for obviousness of the claims challenged in Ground 1, and teaches subject matter directly related to dependent claims 8-9, 13, 20-21 and 24. For these dependent claims, there are two distinct sets: The first set of claims, claims 8, 9, 20 and 21, recite limitations relating to the length of the “plurality of non-random polynucleotide sequences.” Fluidigm does not teach the length of the sequences expressly. However, Fluidigm does suggest targeting fetal DNA in maternal blood samples. Sequenom discloses that fetal DNA in maternal blood samples was known to be of a length the meets the limitations of claims 8, 9, 20 and 21. Sequenom further teaches targeting sequences that are between 10 and 500 base

pairs and 50 and 150 base pairs in length.

185. The second set of claims, claims 13 and 24, recites that the “chromosome tested is selected from the group consisting of chromosome 13, chromosome 18, chromosome 21, chromosome X, and chromosome Y.” While Fluidigm expressly discloses testing for chromosomal aneuploidy, Fluidigm does not disclose particular chromosomes to test. Sequenom is cited because it expressly identifies trisomy 13, 18 and 21 as types of chromosomal aneuploidy that are desirably diagnosed using fetal DNA found in maternal blood.

Rationale for the Combination

186. As I discuss above in ¶¶93-182, it is my opinion that claims 1-7, 10, 14-19, 22 of the '831 Patent would have been obvious over Fluidigm. Sequenom supports my opinion of obviousness, because it demonstrates the well-known use of cell-free DNA from maternal plasma (Ex. 1005, 1:31-2:5, 5:5-13, 8:2-5, 10:26-11:20) for pre-natal diagnostic testing for chromosomal aneuploidy. (Ex. 1005, 10:26-11:20, 24:23-26:27). For example, Sequenom states that:

“Thus the present invention in part features methods of **detecting abnormalities in a fetus by detecting fetal DNA in a biological sample obtained from a mother....**Exemplary diseases that may be diagnosed include, for example, preeclampsia, preterm labor, hyperemesis gravidarum, ectopic pregnancy, **fetal chromosomal aneuploidy (such as trisomy 18, 21, or 13)**, and intrauterine growth retardation.”

(Ex. 1005, 25:10-20)(Emphases added). This supports the motivation (already expressly present in Fluidigm) to use Fluidigm's methods of library preparation on cell-free fetal and maternal DNA to target sequences on a chromosome being tested for aneuploidy.

187. Sequenom also provides specific motivation for dependent claims 8-9, 13, 20-21 and 24, which will be discussed under each claim below.

Graham Factors

188. The four Graham factors (*see* ¶84) are the same as discussed under Ground 1, except that the scope and content of the prior art and differences between the prior art as relevant to claims challenged in Ground 2 are discussed in Ground 2.

Reasonable Expectation of Success

189. In my opinion, by the relevant timeframe one of ordinary skill would have expected reasonable success in combining Sequenom with Fluidigm. The discussion under Ground 1 in ¶¶113-117 is also relevant here. In the combination, Sequenom is cited primarily as disclosing the knowledge of one of ordinary skill concerning the length characteristics of cell-free fetal DNA in maternal blood, the nature of certain types of aneuploidy (*e.g.* trisomy 21), and the desire to test for them. One of ordinary skill would have been able to target DNA sequences of certain lengths by introducing specific forward and reverse primers separated by a region of interest as discussed by Sequenom.

Sequenom is Analogous Art

190. Sequenom is analogous art because it is in the same field as the '831 Patent (DNA sequencing library preparation), and its methods of library preparation would have been reasonably pertinent to the problems facing the named inventors.

Claim Mapping

Claims 1-7, 10, 14-19, and 22

191. Claims 1-7, 10, 14-19, and 22 of the '831 Patent are mapped as discussed in Ground 1, above, ¶¶118-182.

Claims 8-9 and 20-21

192. I have reviewed claims 8 and 20 of the '831 Patent, which appear to be of the same scope except that claim 8 depends from claim 1, while claim 20 depends from claim 14. Because the difference in dependency makes no difference to the analysis, I will analyze claims 8 and 20 together.

“8 [20]. The method of claim 1 [14], wherein each of said plurality of non-random polynucleotide sequences is from 10 to 500 nucleotide bases in length.”

193. As discussed above in ¶¶119-122, Fluidigm renders obvious using cell-free fetal DNA in maternal blood samples. Furthermore, Sequenom teaches the use of cell-free fetal DNA in a maternal plasma sample to test for aneuploidy (¶156, above).

194. As noted in above (¶¶184-156), Sequenom teaches that fetal DNA in

maternal blood samples:

“is relatively small (**approximately 500 base pairs or less**), whereas the majority of circulatory, extracellular maternal DNA in maternal plasma is greater than approximately 500 base pairs.”

(Ex. 1005, 2:1-4)(Emphasis added). Thus, it would have been unlikely to target sequences longer than 500 base pairs to achieve success PCR products when targeting both fetal and maternal cell-free DNA.

195. Sequenom also teaches that it is preferable when testing fetal DNA to target small sequences. (Ex. 1005, 2:2-12). For example, Sequenom teaches that:

“Methods of the present invention allow for the selective enrichment of any **nucleic acid less than a given size** based on the placement of the outside primers. For example, outside primers designed to anneal to a non-target nucleic acid 500 base pair apart often will allow for the **preferential amplification of target nucleic acid less than 500 base pair**. In one embodiment of the invention, methods provided herein are used to preferentially amplify nucleic acid within the range of about 25 bases to about 10,000 bases from a sample comprising a background of longer nucleic 20 acid. In certain embodiments, **the target nucleic acid is at least about 75 base pairs**, but less than about 1200 base pairs. **In some embodiments, the target nucleic acid is less than 500 base pairs.**”

(Ex. 1005, 3:14-21)(Emphases added).

196. Sequenom further teaches using inside primers to target sequences having smaller lengths (that fall within the claimed ranges):

“In some embodiments of the invention, the inside primers flank a locus of interest. In certain embodiments, the pair of inside primers are less than **10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200, 250, 300, 350, 400, 450, 500**, 550, 600, 650, 700, 800, 900, 1 000, 1500 or more base pairs apart.”

(Ex. 1005, 3:22-25)(Emphasis added – bold: claims 8 and 20; bold underlined: claims 9 and 21). Using inside primers in this manner will result in sequences of the targeted lengths. In one example, Sequenom teaches using inside primers to target a sequence of 120 base pairs. (Ex. 1005, 30:6, et seq.). Such use of inside primers to target sequences of a particular length was well-known in the art and could have been adapted with a reasonable expectation of success in Fluidigm’s Modular Approach (within the 9,216 primer pairs).

197. It would have been obvious in view of Sequenom to have each of the plurality of non-random polynucleotide sequences in Fluidigm be from 10 to 500 nucleotide bases in length. Specifically, by suggesting targeting fetal DNA in maternal blood, Fluidigm is already suggesting targeting short (generally under 500 base pair) sequences. Furthermore, Sequenom teaches that it is advantageous to target short sequences. (Ex. 1005, 2:2-12). By adopting Sequenom’s specific suggestion of using inside primers 10, 20, 30, 40, 50, 60, 70, 80, 90, 100, 150, 200,

250, 300, 350, 400, 450, or 500 base pairs apart, the combination would meet claims 8 and 20.

Claims 9 and 21

198. Claims 9 and 21 of the '831 Patent are identical except for their dependencies. Claims 9 and 21 restrict the range of base-pair lengths in claims 8 and 20, respectively, to 50 to 150 base pairs. In my opinion, the combination of Fluidigm and Sequenom would render claims 9 and 21 obvious for the same reasons discussed in ¶¶192-197 with respect to claims 8 and 20, above.

Claims 13 and 24

199. In my opinion, claims 13 and 24 of the '831 Patent would also have been obvious in view of Fluidigm and Sequenom. Claims 13 and 24 are identical, except that claim 13 depends from claim 1, while claim 24 depends from claim 14. Because the difference in dependency makes no difference to the analysis, claims 13 and 24 are addressed together.

“13 [24]. The method of claim 1 [14], wherein said chromosome tested is selected from the group consisting of chromosome 13, chromosome 18, chromosome 21, chromosome X, and chromosome Y.”

200. As discussed above in ¶¶113-116, 119-122 and 130-134, Fluidigm teaches that its methods can be applied to fetal DNA in maternal blood and can be used to detect chromosomal aneuploidy. Sequenom expressly identifies trisomy 13,

18 and 21 (the latter being associated with Down syndrome) as types of chromosomal aneuploidy that can be detected by examining cell-free fetal DNA in maternal blood. (Ex. 1005, 2:10-21, 9:9-18). Sequenom states that, for example:

“[T]he present invention in part features methods of **detecting abnormalities in a fetus by detecting fetal DNA in a biological sample obtained from a mother**. The methods according to the present invention in part provide for detecting fetal DNA in a maternal sample by preferentially amplifying fetal DNA in a background of maternal DNA based on DNA characteristics (e.g., size). See Chan et al. Clin Chem. 2004 Jan;50(1):88-92; and Li et al. Clin Chem. 2004 Jun;50(6):1002-11. Employing such methods, fetal DNA can be predictive of a genetic anomaly or genetic-based disease. These methods are applicable to any and all pregnancy-associated conditions for which nucleic acid changes, mutations or other characteristics (e.g., methylation state) are associated with a disease state. **Exemplary diseases that may be diagnosed include**, for example, preeclampsia, preterm labor, hyperemesis gravidarum, ectopic pregnancy, **fetal chromosomal aneuploidy (such as trisomy 18, 21, or 13)**, and intrauterine growth retardation.”

(Ex. 1005, 25:10-20)(Emphases added). Sequenom specifically teaches using cell-free fetal and maternal DNA from maternal plasma (which is the liquid and cell-free component of blood). (Ex. 1005, 1:31-2:5, 5:5-13, 5:29-6:8, 8:2-5, 10:26-11:20).

DNA in maternal plasma belongs to both the mother and the fetus and is cell-free.

201. Furthermore, in the relevant timeframe, it was well-known that trisomy 21 (also called Down syndrome) was routinely tested in pregnant women. (Ex. 1006, p. 001)(Ex. 1013, p. 001). Accordingly, one of ordinary skill would have been motivated to test for at least trisomy 21 using the method taught in Fluidigm based on the disclosure by Sequenom and the knowledge in the art.

Ground 3. Claim 12 is obvious as in Ground 1 in further view of Harismendy

202. In my opinion Claim 12 of the '831 Patent would have been obvious over Fluidigm and the knowledge of one of ordinary skill, as presented in ¶¶93-182, above, in further view of Harismendy. (Ex. 1007)(“Harismendy”).

Overview of Claim 12

203. Claim 12 recites:

“The method of claim 1, wherein said non-random polynucleotide sequences comprise sequences that are sequenced at a rate of greater than 5-fold than other sequences on the chromosome.”

204. Claim 12 appears to relate to the '831 Patent's discussion of “hotspots.” According to the '831 Patent, a hotspot is a portion of the genome that, when sequenced, appears at a higher frequency (*i.e.* number of unique sequence reads) than other sequences. The '831 Patent describes that:

“Selecting Sequences Based on ‘Hotspots’ [¶] Sequencing data

can be analyzed to identify polynucleotide sequences to be selectively enriched. **Some polynucleotide sequences** from a sample comprising nucleic acids (e.g., genomic DNA) **can be sequenced at a higher frequency** than other polynucleotide sequences. These sequences may be more likely to be enriched by, for example, amplification methods.”

(Ex. 1001, 6:59-65)(Emphases added). The ’831 Patent suggests targeting these high-frequency regions (“hotspots”) to reduce the cost of the process:

“Identifying and enriching these polynucleotide sequences can reduce the number of nucleic acids that need to be analyzed to determine the presence or absence of fetal aneuploidy. This enrichment can reduce the cost of aneuploidy determination.”

(Ex. 1001, 6:65-7:3)

205. The ’831 Patent does not, however, explain how to identify hotspot regions, but rather merely states that frequency rate data can be obtained from an unidentified database:

“In one embodiment, the non-random sequences to be selectively enriched are identified based on the number of times they are sequenced in a database of sequence information, independent of the rate of sequencing of a second set of sequences.”

(Ex. 1001, 8:16-20; 7:10-22).

206. The sequencing rate is thus the so-called “coverage” of a DNA sequence in a database. (Ex. 1001, 3:34-36)(“Sequences for enrichment can be

chosen on the basis of being in a ‘hotspot,’ a region of relatively high sequence coverage.”) The term “coverage” means the number of unique sequence reads at a given location (base pair) on a chromosome. The ’831 Patent “hotspot” strategy thus essentially entails looking in a database that is not described at all to determine the coverage of various sequences and selecting those sequences with high coverage.

207. Claim 12 also does not require any particular strategy for selecting sequences. In claim 12, “sequences” (*i.e.* two or more sequences) among the at least 100 sequences required by claim 1 must be “sequenced at a rate of greater than 5-fold than other sequences” (*i.e.* two other sequences) on the same chromosome.

Overview of the Combination

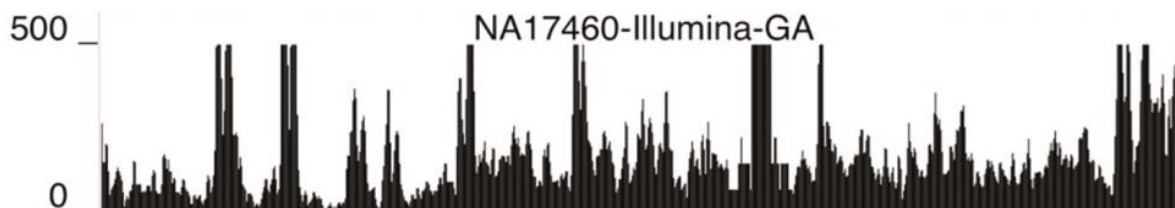
208. In my opinion, Harismendy demonstrates that it would have been obvious to target portions of the genome that sequence at a five-fold greater rate than other sequences on the same chromosome. Harismendy studied and compared different NGS platforms. With each of the platforms, Harismendy observed the hotspot phenomenon:

“Interestingly, **each NGS technology has a unique reproducible pattern of non-uniform sequence coverage:** sequences with high or low coverage in one sample typically had high or low coverage in the other three samples (Fig. 3).”

(Ex. 1007, p. 5)(Emphasis added).

209. In Figure 2 of Harismendy, there is shown the variation of coverage

(fold) for various different sequencing platforms across a 100-kb portion of chromosome 3. (Ex. 1007, p. 4, Fig. 2, caption). A portion of Fig. 2, reproduced below, shows non-uniform sequence coverage for an approximately 17-kb region of chromosome 3 when using the Illumina Genome Analyzer (“GA”) system:



(Ex. 1007, p. 4, Fig. 2). In the Figure, the y-axis is the “fold” coverage scale, while the x-axis reflects a position on the chromosome. (Ex. 1007, p. 4, Fig. 2, caption). As can be seen in the full figure 2, the fold varies across the gene from near-zero to well into the thousands (in the portions shown above, several peaks are off the top of the scale).

210. I note that similar variations have been observed by others in connection with other chromosomes. For example, Fan, *et al.* (Ex. 1006), in the process of testing for trisomy 21, “observed a nonuniform distribution of sequence tags across each chromosome. This pattern of intrachromosomal variation was common among all samples, including randomly sheared genomic DNA, indicating that the observed variation was most probably due to sequencing artifacts.” (Ex. 1006, p. 001).

Rationale for the Combination

211. In my opinion, it would have been obvious to target high-coverage regions of a chromosome in Fluidigm's method based on Harismendy.

212. Harismendy notes that each NGS platform tested has its own pattern of coverage. (Ex. 1007, p. 5). Fluidigm teaches using its methods to prepare libraries for later Solexa sequencing. "Solexa" refers to a company that was later purchased by Illumina. After the purchase, Illumina began selling Solexa's Genome Analyzer product. (Ex. 1014).

213. Harismendy teaches that the Illumina GA sequencer performs best when the sequence coverage is high, and worst at low coverage:

“[T]he performance of the NGS technologies at low sequence coverage is correlated with per-base sequence coverage uniformity; the **Illumina GA**, which has the highest coverage variability, **performs the worst at lower coverage....**”

(Ex. 1007, p. 8, right column)(Emphases added). Harismendy also reports that:

“**At high sequence coverage all NGS platforms have excellent variant calling accuracy (>95%)** as assessed by the detection of known SNP variants.”

(Ex. 1007, p. 9, left column, top)(Emphasis added)

214. To increase the accuracy of the Illumina GA sequencer, it would have been obvious in my opinion to target regions of a chromosome with high sequence

coverage. To do this, one of ordinary skill would have obviously chosen at least two of (and preferably all of) Fluidigm's 9,216 primer pairs to anneal to sequences on a chromosome that at higher coverage than other sequences. Furthermore, because the coverage varies by thousands across a chromosome (as indicated in Harismendy—see Fig. 3), it would have been obvious to have at least two sequences that are sequenced at a rate of greater than 5-fold than other sequences on the chromosome. (Ex. 1007, pp. 003-004)(e.g., “For all three NGS technologies there is greater than a hundred-fold variation in the per base sequence coverage depth (Figure 2).”

215. Finally, selecting similarly high-coverage sequences would have lowered the variation in coverage, thus reducing the total amount of sequence generation required. (Ex. 1007, p. 9, left column bottom)(“[T]he uniformity of per-base sequence coverage must be improved to reduce the total amount of sequence generation required....”).

Graham Factors

216. The *Graham* factors (see ¶84, above) are the same as discussed under Ground 1, except that the scope and content of the prior art and differences between the prior art as relevant to claims challenged in Ground 3 are discussed in Ground 3.

Reasonable Expectation of Success

217. One of ordinary skill would have expected reasonable success in

combining Harismendy with Fluidigm. In the combination, Harismendy is cited primarily as describing the knowledge of one of ordinary skill concerning the non-uniform coverage with some regions having >5-fold coverage. Selecting these sequences would simply have been a matter of obtaining the available coverage data or sequencing portions of the chromosomes to be tested, both of which were well within ordinary skill in the relevant timeframe.

Harismendy is Analogous Art

218. Harismendy is analogous art at least because it is directed to DNA sequencing, and thus reasonably pertinent to problems faced by the '831 Patent named inventors (DNA sequencing library preparation).

Claim Mapping

219. As discussed above, it was obvious to target, as part of Fluidigm's Modular Approach using 9,216 target sequences, at least two sequences that are sequenced at a rate of greater than 5-fold than other sequences on the chromosome based on Harismendy. The combination of Harismendy with Fluidigm meets the requirement of claim 12 of the '831 Patent.

Ground 4. Claims 12 are obvious as in Ground 2 in further view of Harismendy

Ground 4 is the same as Ground 3 but based on Ground 2 instead of Ground 1. The discussion under Ground 3 applies in the same way to Ground 2 as to Ground

1. As in Ground 2, Sequenom provides additional support for obviousness.

XII. OATH

220. This declaration and my opinions herein are made to the best of my knowledge and understanding, and based on the material available to me, at the time of signing this declaration. I declare under penalty of perjury under the laws of the United States of America that all statements made of my own knowledge are true and that all statements made on information and belief are believed to be true. I understand that willful false statements and the like are punishable by fine or imprisonment, or both (18 U.S.C. §1001) and may jeopardize the validity of the application or any patent issuing thereon.

Date: June 29, 2018

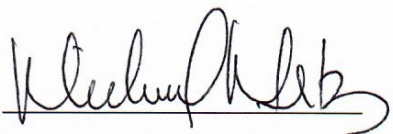
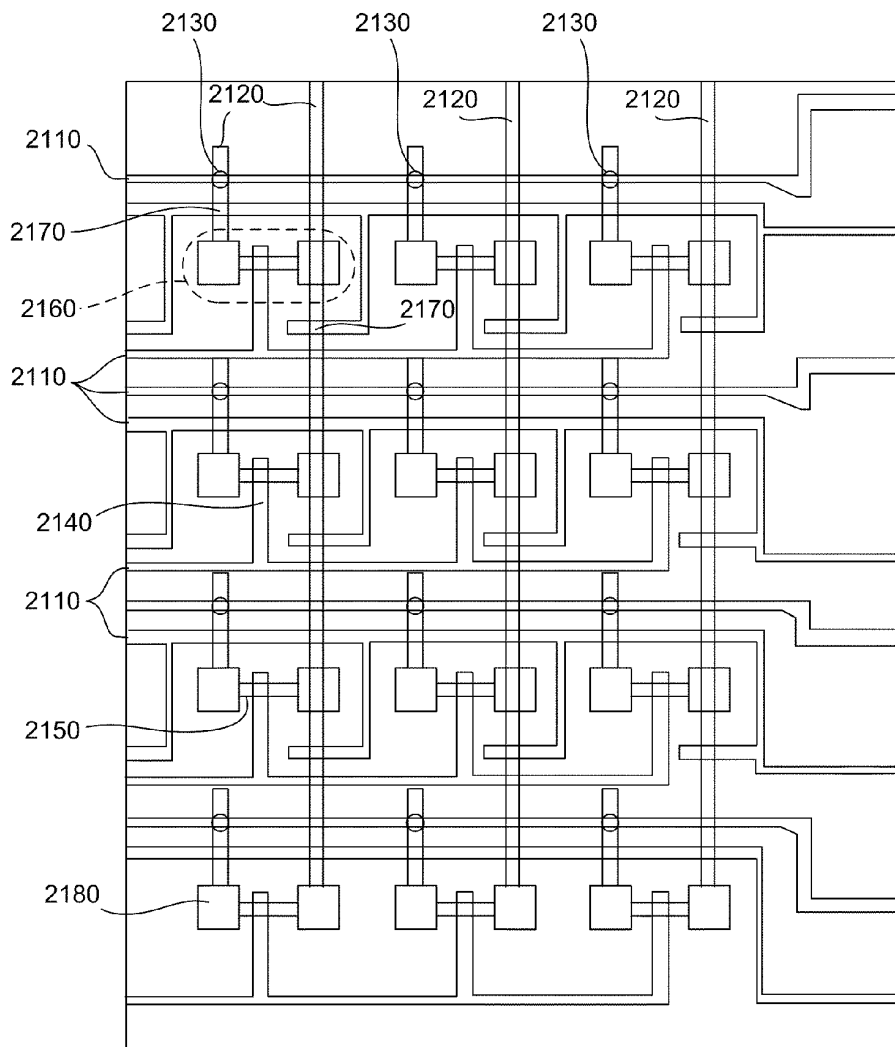
Signed: 

Exhibit 13

(19) **United States**(12) **Patent Application Publication**
Mir et al.(10) **Pub. No.: US 2010/0120038 A1**(43) **Pub. Date: May 13, 2010**(54) **ASSAY METHODS FOR INCREASED
THROUGHPUT OF SAMPLES AND/OR
TARGETS**(22) Filed: **Aug. 26, 2009****Related U.S. Application Data**(75) Inventors: **Alain Mir**, Cupertino, CA (US);
Ramesh Ramakrishnan, San Jose,
CA (US); **Marc Unger**, San Mateo,
CA (US); **Bernhard G.
Zimmermann**, San Mateo, CA
(US)(60) Provisional application No. 61/092,010, filed on Aug.
26, 2008, provisional application No. 61/098,621,
filed on Sep. 19, 2008, provisional application No.
61/146,567, filed on Jan. 22, 2009.**Publication Classification**(51) **Int. Cl.**
C12Q 1/68 (2006.01)
G01N 33/48 (2006.01)(52) **U.S. Cl.** **435/6; 436/94**(57) **ABSTRACT**

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Weaver Austin Villeneuve & Sampson LLP
P.O. BOX 70250
OAKLAND, CA 94612-0250 (US)(73) Assignee: **Fluidigm Corporation**(21) Appl. No.: **12/548,132**The present invention provides assay methods that increase
the number of samples and/or target nucleic acids that can be
analyzed in a single assay.

NATERA 1003

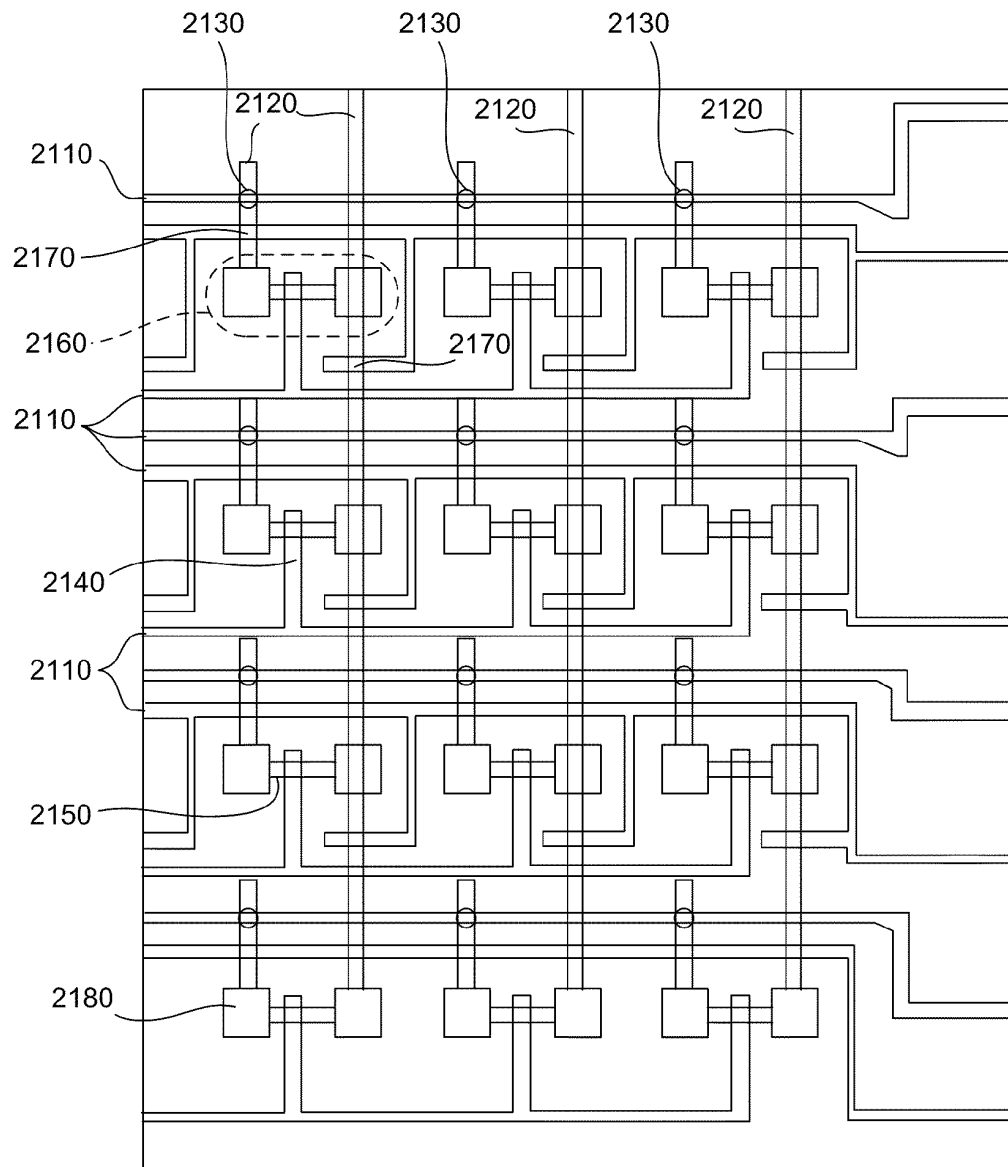
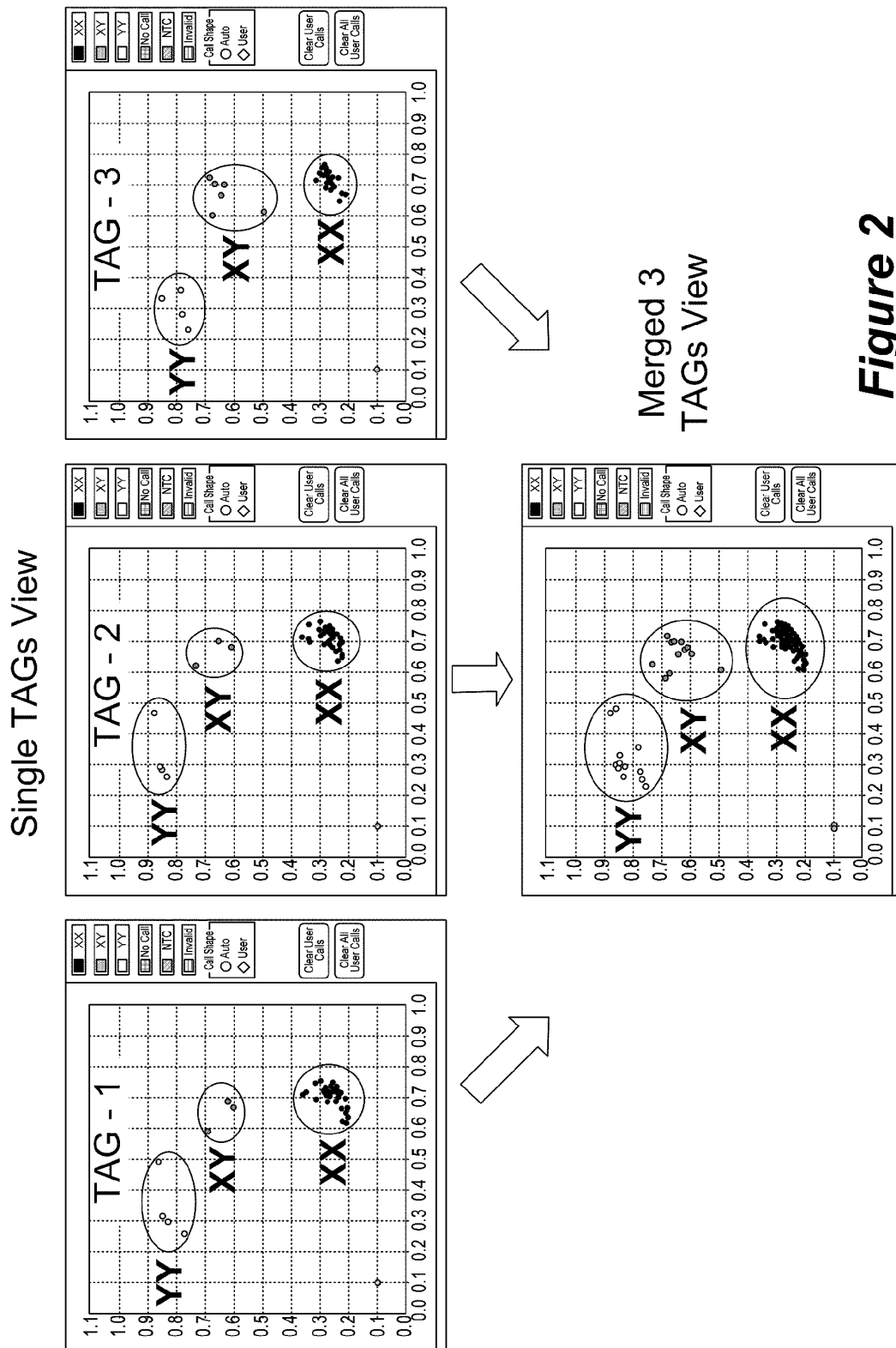


Figure 1



Truth (not Tagged)

Assay	% Call Rate, n=3	
	% Call Rate	SD
SNP01	96.4	1.3
SNP02	97.8	0.0
SNP03	97.8	0.0
SNP04	97.1	1.3
SNP05	97.1	1.3
SNP06	97.8	0.0
SNP07	97.8	0.0
SNP08	97.8	0.0
SNP09	97.8	0.0
SNP10	98.6	1.3
SNP11	97.1	1.3
SNP12	97.8	0.0
SNP13	95.7	0.0
SNP14	95.7	0.0
SNP15	97.8	0.0
SNP16	97.8	0.0
Average	97.4	0.4

Std Genotyping (no tagging)**Invalids, no calls removed****Mean Call rate = 97.4%****Test (Tagged)**

Assay	% Call Rate by Tag		
	1	2	3
SNP01	100	100	97.9
SNP02	100	100	97.9
SNP03	100	100	97.9
SNP04	100	100	97.9
SNP05	100	100	100
SNP06	100	100	100
SNP07	100	100	100
SNP08	100	100	98
SNP09	100	100	100
SNP10	100	100	97.9
SNP11	100	100	97.9
SNP12	100	100	97.9
SNP13	100	100	97.9
SNP14	100	100	100
SNP15	100	100	100
SNP16	100	100	100
Average	100	100	98.8

Tagged Genotyping**Same DNA, Same assays****Mean call rate 98.8 - 100%****Figure 3**

Tagging Reaction

Tagging reaction assay Forward primer bears a 5' Tag sequence.



Reaction in Chip

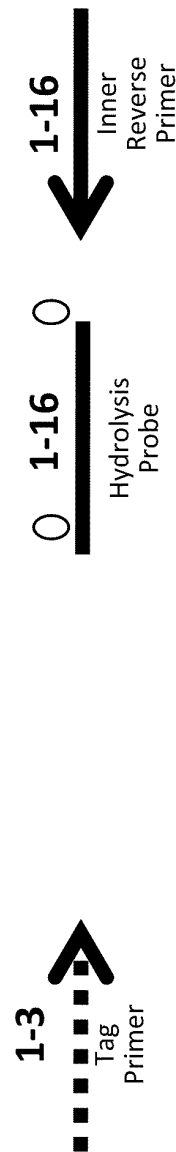


Figure 4

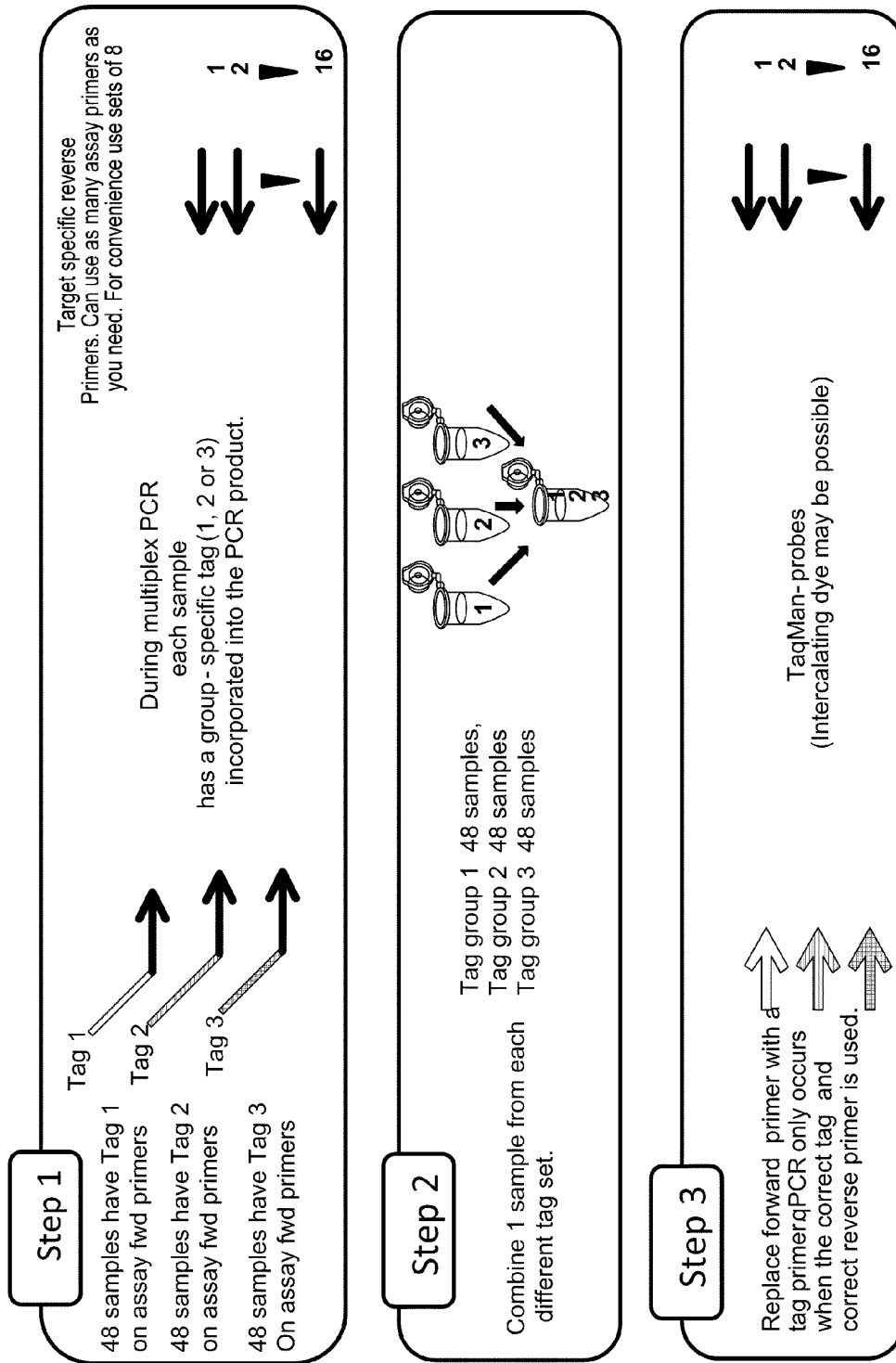


Figure 5

- Prepare 3 encoding primer sets

- Each is a mixture of 32 primers

- For example, here is Encoding Primer Set 1:

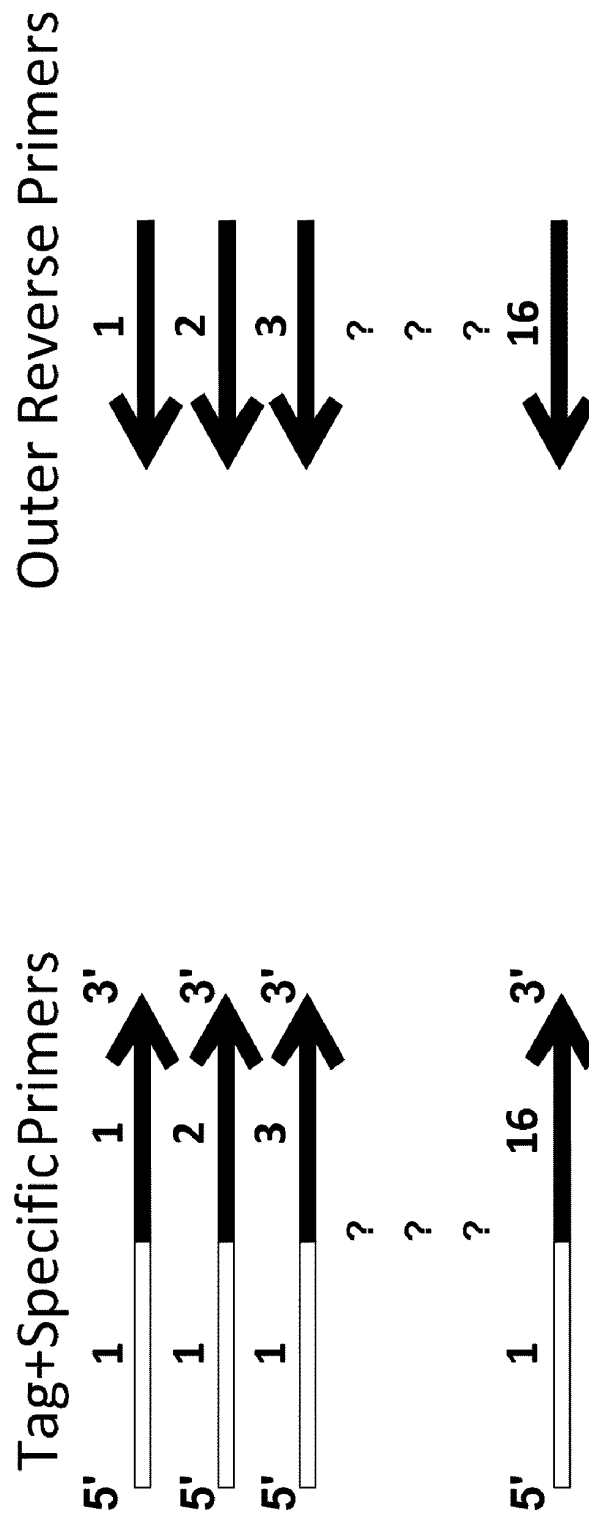


Figure 6

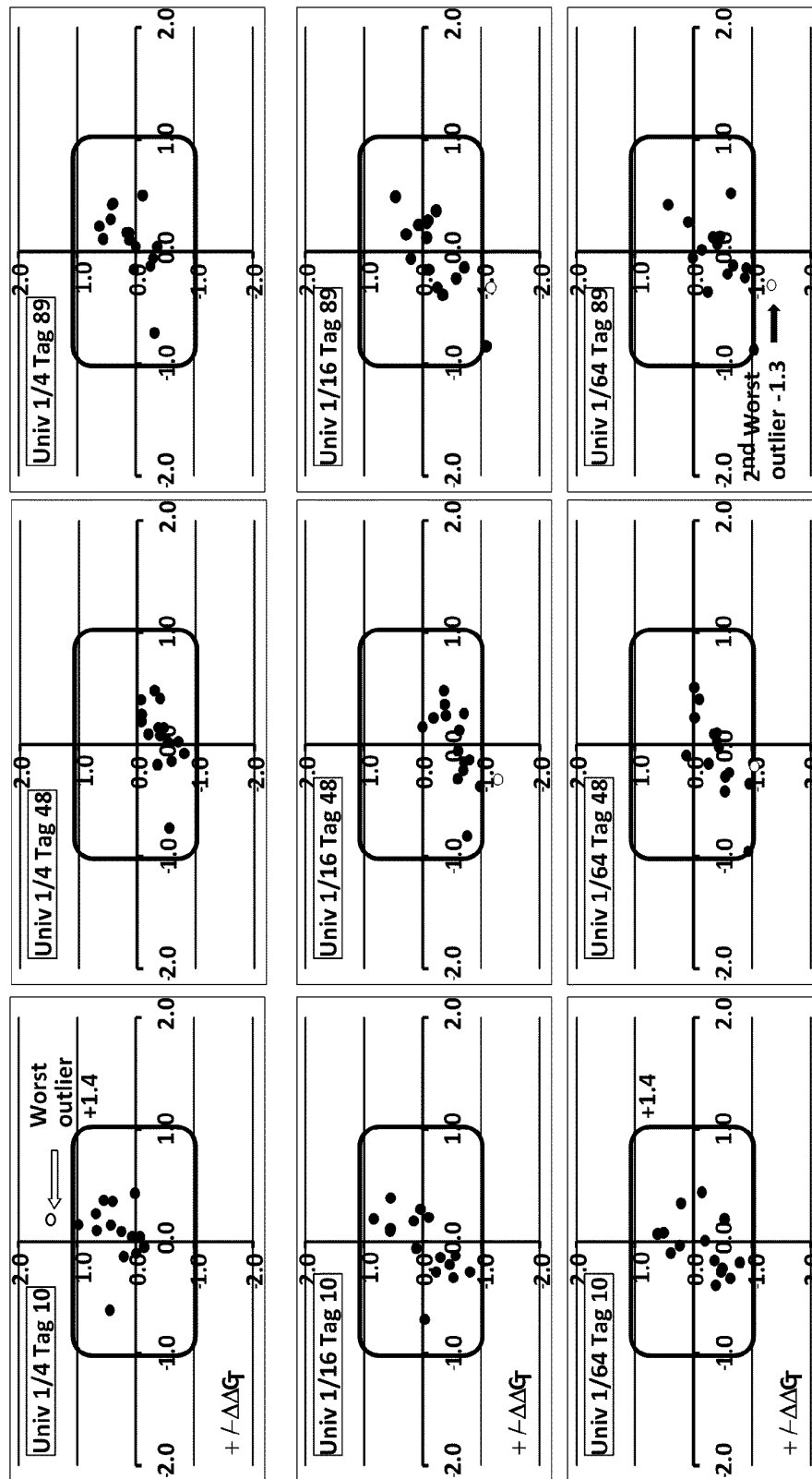


Figure 7

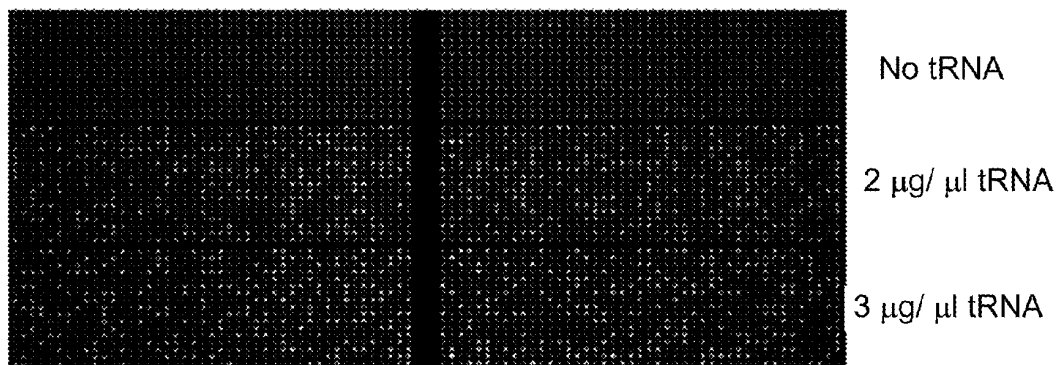


Figure 8

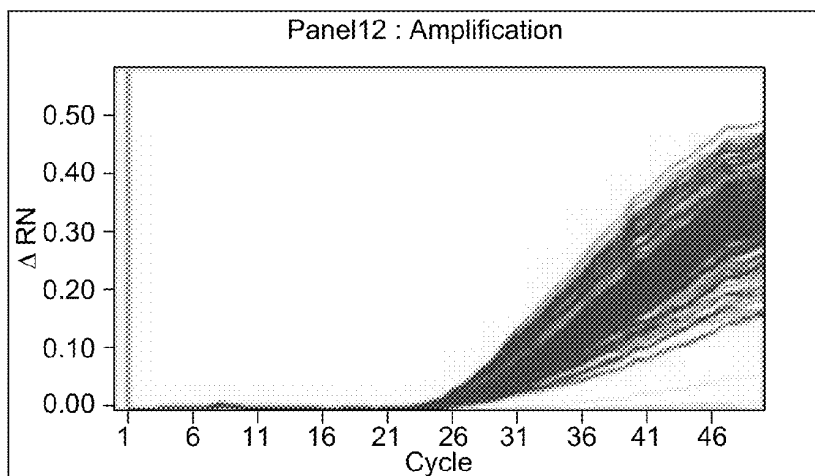
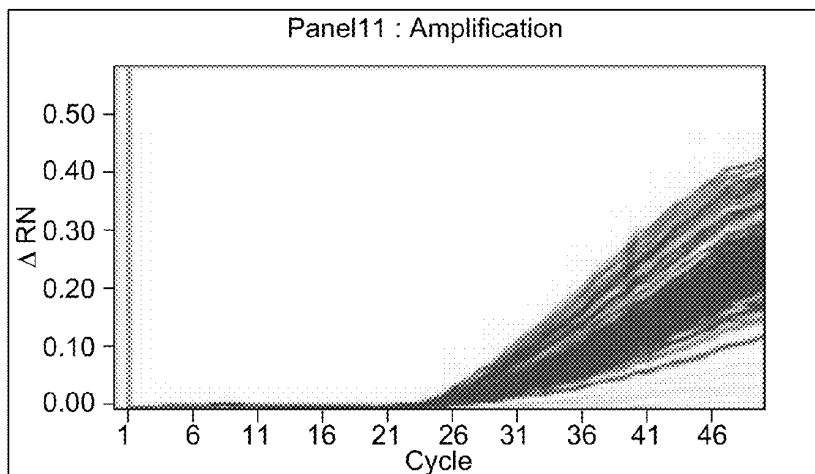
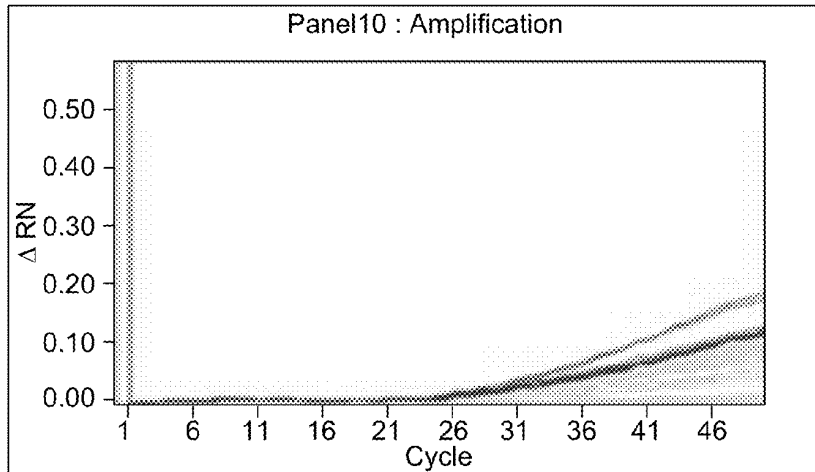


Figure 9

US 2010/0120038 A1

May 13, 2010

1

ASSAY METHODS FOR INCREASED THROUGHPUT OF SAMPLES AND/OR TARGETS

CROSS-REFERENCE TO RELATED APPLICATIONS

[0001] This application hereby incorporates prior U.S. provisional application Nos. 61/092,010, filed Aug. 26, 2008; 61/098,621, filed Sep. 19, 2008; and 61/146,567, filed Jan. 22, 2009 by reference in their entireties.

FIELD OF THE INVENTION

[0002] The present invention relates generally to the area of high-throughput assays for detection of particular target nucleic acids. In particular, the invention relates to methods for increasing the number of samples and/or targets that can be analyzed in a single assay.

BACKGROUND OF THE INVENTION

[0003] The ability to detect specific nucleic acid sequences in a sample has resulted in new approaches in diagnostic and predictive medicine, environmental, food and agricultural monitoring, molecular biology research, and many other fields.

[0004] Additional methods, especially methods that allow detection of many targets and/or the analysis of many samples simultaneously across a broad range of concentrations in a sample would be of great benefit.

SUMMARY OF THE INVENTION

[0005] The invention provides a first assay method for detecting a plurality of target nucleic acids (i.e., T target nucleic acids, where T is an integer greater than one) in a plurality of samples. In certain embodiments, the method entails providing S samples that will be mixed together prior to assay, where S is an integer greater than 1. Each of these samples can be separately subjected to an encoding reaction that produces a set of T tagged target nucleotide sequences, each tagged target nucleotide sequence comprising a sample-specific nucleotide tag and a target nucleotide sequence. For each of these S samples (i.e., each of the samples to be combined), the tagged target nucleotide sequences can be mixed to form an assay mixture. In this manner, samples can be assayed in batches, so that, if, e.g., 48 samples are to be analyzed, S can be, e.g., 3, which means that 16 assay mixtures are prepared. See Example 1. The assay mixture, or aliquots thereof, can be subjected to amplification using S×T unique pairs of amplification primers, wherein each amplification primer pair comprises:

[0006] a forward or a reverse amplification primer that anneals to a target nucleotide sequence; and

[0007] a reverse or a forward amplification primer, respectively, that anneals to a sample-specific nucleotide tag. For each unique primer pair, the presence or, in particular embodiments, amount an amplification product is in the amplification mixture, or aliquot thereof, is determined. The presence or amount of an amplification product indicates the presence or amount of a particular target nucleic acid in a particular sample.

[0008] In illustrative embodiments of the first assay method, the encoding reaction comprises separately subjecting each of the S samples to preamplification using a distinct

set of forward and reverse preamplification primers for each sample to produce preamplified samples, wherein:

[0009] each preamplification primer set comprises T pairs of forward and reverse preamplification primers, wherein each preamplification primer pair is capable of amplifying a particular target nucleic acid; and

[0010] either all forward preamplification primers or all reverse preamplification primers in a given set comprise a common sample-specific nucleotide tag.

The preamplified samples for each of the S samples are mixed to form an assay mixture (e.g., one assay mixture for each set of samples to be analyzed together). The assay mixture can then be analyzed by dividing it into up to S×T amplification mixtures, and separately subjecting each of the amplification mixtures to amplification using a unique pair of amplification primers, wherein each amplification primer pair comprises:

[0011] a forward or a reverse amplification primer that anneals to a target nucleotide sequence; and

[0012] a reverse or a forward amplification primer, respectively, that anneals to a sample-specific nucleotide tag.

The presence or amount of an amplification product in the amplification mixtures is determined to determine the presence or amount of a particular target nucleic acid in a particular sample.

[0013] The invention provides a second assay method for detecting a plurality of target nucleic acids (i.e., T target nucleic acids, where T is an integer greater than one) in a plurality of samples. In certain embodiments, the method entails providing S samples that will be mixed together prior to assay, where S is an integer greater than 1. Each of these samples is separately subjected to an encoding reaction that produces a set of T tagged target nucleotide sequences, each tagged target nucleotide sequence comprising a first nucleotide tag linked to a target nucleotide sequence, which is linked to a second nucleotide tag. For each of these S samples (i.e., each of the samples to be combined), the tagged target nucleotide sequences are mixed to form an assay mixture. The assay mixture, or aliquots thereof, is subjected to amplification using S×T unique pairs of amplification primers, wherein each amplification primer pair comprises:

[0014] a forward or a reverse amplification primer that anneals to a first nucleotide tag; and

[0015] a reverse or a forward amplification primer, respectively, that anneals to a second nucleotide tag.

For each unique primer pair, the presence or amount of an amplification product in the amplification mixture, or aliquot thereof, is determined. The presence of an amplification product indicates the presence or amount of a particular target nucleic acid in a particular sample.

[0016] In exemplary embodiments of the second assay method, the encoding reaction comprises separately subjecting each of the S samples to preamplification using a distinct set of forward and reverse preamplification primers for each sample to produce preamplified samples, wherein:

[0017] each preamplification primer set comprises T pairs of forward and reverse preamplification primers, wherein each preamplification primer pair is capable of amplifying a particular target nucleic acid; and

[0018] each forward preamplification primer comprises a forward nucleotide tag, and each reverse preamplification primer comprises a reverse nucleotide tag;

The preamplified samples for each of the S samples are mixed to form an assay mixture. The assay mixture can then be analyzed by dividing each assay mixture into up to S×T

amplification mixtures, and separately subjecting each of the amplification mixtures to amplification using a unique pair of amplification primers, wherein each amplification primer pair comprises:

[0019] a forward amplification primer that anneals to a forward nucleotide tag; and

[0020] a reverse amplification primer that anneals to a reverse nucleotide tag.

The presence or amount of an amplification product present in the amplification mixtures is determined. The presence of an amplification product indicates the presence or amount of a particular target nucleic acid in a particular sample.

[0021] The invention also provides a third assay method, which detects a plurality of target nucleic acids (i.e., T target nucleic acids) in a sample. The method entails providing T forward preamplification primers to a sample, wherein each forward preamplification primer comprises a different target-specific nucleotide sequence and a set-specific nucleotide tag, wherein X different forward set-specific nucleotide tags are employed, and X is an integer that is greater than 1 and less than T, whereby T/X primers comprise the same forward set-specific nucleotide tag. Also provided to the sample are T reverse preamplification primers, wherein each reverse preamplification primer comprises a different target-specific nucleotide sequence and a reverse set-specific nucleotide tag, wherein Y different reverse set-specific nucleotide tags are employed, and Y is an integer that is greater than 1 and less than T, whereby T/Y primers comprise the same reverse set-specific nucleotide tag. The sample is subjected to preamplification to produce an assay mixture, wherein any preamplification product produced for a particular target incorporates a unique combination of forward and reverse set-specific nucleotide tags. The assay mixture, or aliquots thereof, to amplification using amplification primers wherein each amplification primer pair comprises:

[0022] a forward amplification primer that anneals to the forward set-specific nucleotide tag; and

[0023] a reverse amplification primer that anneals to the reverse set-specific nucleotide tag. For each unique primer pair, the presence or amount of an amplification product in the amplification mixture, or aliquot thereof, is determined. The presence of an amplification product indicates the presence of a particular target nucleic acid in the sample.

[0024] A fourth assay method of the invention provides a modular approach to detecting a plurality target nucleic acids in a sample. This method entails dividing a sample into R aliquots, wherein R is an integer greater than 1. Each of the R aliquots is separately subjected to an encoding reaction that produces a set of T tagged target nucleotide sequences, wherein T is the number of target nucleic acids to be detected in each aliquot, T being an integer greater than 1. Each tagged target nucleotide sequence comprises a first nucleotide tag 5' of a target nucleotide sequence, a target nucleotide sequence, and a second nucleotide tag 3' of the target nucleotide sequence. The combination of nucleotide tags in each of said T tagged target nucleotide sequences is unique for every tagged target nucleotide sequence in each aliquot. However, the same set of first and second nucleotide tag combinations is used in the encoding reaction in each of the aliquots. This aspect of the method makes it possible to separately subject each aliquot to amplification using the same set of T different amplification primer pairs for each aliquot. Each primer pair includes a first primer that anneals to the first nucleotide tag and a second primer that anneals to the second nucleotide tag

in each tagged target nucleotide sequence. For each unique primer pair in each aliquot, the method entails determining whether an amplification product is present in the aliquot. The presence of an amplification product indicates the presence of a particular target nucleic acid in the sample.

[0025] In a variation of this method, after the encoding reaction, each aliquot is divided into T sub-aliquots. One of the T different amplification primer pairs is combined with each sub-aliquot, and the sub-aliquots are subjected to separate amplification reactions.

[0026] In particular embodiments of the above-described methods, the sample can be a genomic DNA sample. In variations of these embodiments, preamplification of genomic DNA can be conducted in the presence of an amount of a blocking agent that is sufficient to increase specific amplification of the target nucleic acid.

BRIEF DESCRIPTION OF THE DRAWINGS

[0027] The invention may be understood by reference to the following description taken in conjunction with the accompanying drawings that illustrate certain specific embodiments of the present invention.

[0028] FIG. 1 depicts an exemplary matrix-type microfluidic device plan view.

[0029] FIG. 2 shows a graphical depiction of the results of the application of a method of the invention for analyzing multiple target nucleic acids in multiple samples in a genotyping study (Example 2). Samples were amplified with forward primers bearing 3 different tag sequences. The results are shown in the Single TAG view allelograms (top images) labeled TAG—1, 2, and 3. All samples were clearly differentiated into (circled) homozygote XX, YY or heterozygote XY genotypes. Data from the merged genotypes of these allelograms are shown as “Merged 3”.

[0030] FIG. 3 shows the data from the genotyping study of Example 2. 16 SNP loci were genotyped using tagging and, for comparison, standard methodologies. Untagged samples are described as “Truth.” The same untagged samples shown in the “Truth” table were compared against samples bearing Tag sequences 1, 2, or 3 on the forward primer (“Test”) as described in Example 2. Tagged samples display 1.4-to-2% increase in call rate. All no-called SNPs in Tag 3 were derived from a single sample.

[0031] FIG. 4 shows an illustrative setup for analyzing multiple target nucleic acids in multiple samples as in, e.g., the gene expression study of Example 3. This study employed 3 tag groups (1-3) targeting 16 loci. Three sets of 16 forward primers for amplifying 16 cDNAs were synthesized. If each group tags 48 individuals, and 1 sample from each tag group is combined, 144 samples can be simultaneously analyzed. Before the off-chip tagging reaction, samples were divided into each of the 3 tag sets. Each group of 48 samples bears either Tag sequence 1, 2, or 3 on the 5' termini of the forward primers. Tagging reactions employ an assay-specific tagged primer and an outer reverse primer. On chip, a nested reverse primer can be used. In the example for gene expression, a single hydrolysis probe is shown.

[0032] FIG. 5 shows an illustrative generic setup for analyzing multiple target nucleic acids in multiple samples. For example, allele-specific probes (genotyping) or a single probe (gene expression) can be used. After combining samples, residual primers can be removed using ExoSAP-IT (USB) prior to the PCR step.

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[0033] FIG. 6 shows an illustrative generic setup for analyzing multiple target nucleic acids in multiple samples, in this case, 16 assays are performed on 144 samples. Three sets of 16 forward primers for amplifying 16 loci are synthesized. Each tag set also bears Tag sequence 1, 2, or 3 on the 5' primer termini. During the off-chip tagging reaction, 32 primers consisting of 16 forward assay primers with tag sequence 1 and 16 reverse assay-specific reverse primers are used. Assay-specific tag primers bearing a different 5' tag sequence (Tag 2) and the same sequence reverse primers are used to tag the next set of samples. This is also performed with Tag type 3 and another set of samples. After tagging 48× samples with tag set 1, 48× samples with tag set 2, and 48× samples with tag set 3, one sample per each tag set is combined. This generates 48 mixed samples, and efficient PCR amplification of a specific target from one of the mixed samples (but not the other two) will only occur when the correct tag primer and target specific reverse primer are added to the pooled samples.

[0034] FIG. 7 shows qPCR data derived from 144 serially diluted universal reference cDNA amplified with 3 different tags (Tags 10, 48, and 89) targeting 16 different genes (Example 3). Data are from a single 96.96 array. Since all cDNA samples were dilutions of the same sample, the expected $\Delta\Delta CT$ is 0.96.5% of assays display a $\Delta\Delta CT$ within the highlighted target zone of -1 to +1 cycle. 5-of-144 samples (~3.5%) have a $\Delta\Delta CT$ of 1.0 to 1.4. The two highest outliers with a $\Delta\Delta CT$ of 1.3 and 1.4 are highlighted by arrows. $\Delta\Delta CT$ was calculated by subtracting the control ACT gene (Bax) from test ACT for each tag/sample dilution/assay combination using universal reference cDNA.

[0035] FIG. 8 shows the results of digital PCR on a 12.765 Digital Array commercially available from Fluidigm Corp. (South San Francisco, Calif.). Human genomic DNA was preamplified in the presence of varying amounts of tRNA and then analyzed by digital PCR, as described in Example 4. Specifically, preamplification was performed on human genomic DNA, using protocols described in Qin J., Jones R C, Ramakrishnan R. (2008) *Studying copy number variations using a nanofluidic platform Nucleic Acids Research*, Vol. 36, No. 18 e116 on the GeneAmp PCR system 9700 (Applied Biosystems, CA) in a 25 μ l reaction containing 1× PreAmp master mix (Applied Biosystems, CA), 900 nM primers, ~10 ng of DNA sample and differing amount of tRNA. Samples were diluted and analyzed on the digital array as described in Qin et al. Equal amounts of genomic DNA were used in all panels shown. The upper two panels show the negative controls—preamplification conducted in the absence of tRNA, while the next two pairs of panels show the effects of adding either 2 μ g/ μ l or 3 μ g/ μ l tRNA to the preamplification reaction mix. It is clear that the addition of tRNA increases the intensity of the specific amplification signal and suppresses background.

[0036] FIG. 9 shows the effect of adding tRNA to preamplification reaction mixtures on the quality of specific amplification curve. The plots shown in FIG. 9 are from the experiment described in Example 4 and reflect real time PCR plots from the same chip panels shown in FIG. 8. The first panel shows the amplification plot in the absence of tRNA in the preamplification mix, and the second and third panels show the effect when either 2 μ g/ μ l or 3 μ g/ μ l of tRNA was included in the preamplification reaction mix, respectively. The amplification plots confirm the observation from FIG. 8 that the addition of tRNA increases the total amount of specific amplifiable signal, (increase number of hits) and also

show that the addition of tRNA improves the quality of amplification (possibly by improving the efficiency of PCR).

DETAILED DESCRIPTION

[0037] The present invention provides methods for increasing the number of samples that can be analyzed for one or multiple targets in a single assay, as well as methods for increasing the number of targets that can be analyzed in a sample, while minimizing increases in assay cost. The methods are particularly well-suited for increasing the efficiency of assays performed on matrix-type microfluidic devices.

[0038] It is understood that the invention is not limited to the particular methodology, protocols, and reagents, etc., described herein, as these can be varied by the skilled artisan. It is also understood that the terminology used herein is used for the purpose of describing particular illustrative embodiments only, and is not intended to limit the scope of the invention. It also noted that as used herein and in the appended claims, the singular forms “a,” “an,” and “the” include the plural reference unless the context clearly dictates otherwise. Thus, for example, a reference to “a cell” is a reference to one or more cells and equivalents thereof known to those skilled in the art.

[0039] The embodiments of the invention and the various features and advantageous details thereof are explained more fully with reference to the non-limiting embodiments and examples that are described and/or illustrated in the accompanying drawings and detailed in the following description. It should be noted that the features illustrated in the drawings are not necessarily drawn to scale, and features of one embodiment may be employed with other embodiments as the skilled artisan would recognize, even if not explicitly stated herein. Descriptions of well-known components and processing techniques may be omitted so as to not unnecessarily obscure the embodiments of the invention.

DEFINITIONS

[0040] Terms used in the claims and specification are defined as set forth below unless otherwise specified. These terms are defined specifically for clarity, but all of the definitions are consistent with how a skilled artisan would understand these terms.

[0041] The term “adjacent,” when used herein to refer two nucleotide sequences in a nucleic acid, can refer to nucleotide sequences separated by 0 to about 20 nucleotides, more specifically, in a range of about 1 to about 10 nucleotides, or sequences that directly abut one another. In addition, two primers may be said to be adjacent if they overlap, for example adjacent primers can overlap by about 1 to about 4 nucleotides.

[0042] The term “nucleic acid” refers to a nucleotide polymer, and unless otherwise limited, includes known analogs of natural nucleotides that can function in a similar manner (e.g., hybridize) to naturally occurring nucleotides.

[0043] The term nucleic acid includes any form of DNA or RNA, including, for example, genomic DNA; complementary DNA (cDNA), which is a DNA representation of mRNA, usually obtained by reverse transcription of messenger RNA (mRNA) or by amplification; DNA molecules produced synthetically or by amplification; and mRNA.

[0044] The term nucleic acid encompasses double- or triple-stranded nucleic acid, as well as single-stranded molecules. In double- or triple-stranded nucleic acids, the nucleic

acid strands need not be coextensive (i.e., a double-stranded nucleic acid need not be double-stranded along the entire length of both strands).

[0045] The term nucleic acid also encompasses any chemical modification thereof, such as by methylation and/or by capping. Nucleic acid modifications can include addition of chemical groups that incorporate additional charge, polarizability, hydrogen bonding, electrostatic interaction, and functionality to the individual nucleic acid bases or to the nucleic acid as a whole. Such modifications may include base modifications such as 2'-position sugar modifications, 5-position pyrimidine modifications, 8-position purine modifications, modifications at cytosine exocyclic amines, substitutions of 5-bromo-uracil, backbone modifications, unusual base pairing combinations such as the isobases isocytidine and isoguanidine, and the like.

[0046] More particularly, in certain embodiments, nucleic acids, can include polydeoxyribonucleotides (containing 2-deoxy-D-ribose), polyribonucleotides (containing D-ribose), and any other type of nucleic acid that is an N- or C-glycoside of a purine or pyrimidine base, as well as other polymers containing normucleotidic backbones, for example, polyamide (e.g., peptide nucleic acids (PNAs)) and polymorpholino (commercially available from the Anti-Virals, Inc., Corvallis, Oreg., as Neugene) polymers, and other synthetic sequence-specific nucleic acid polymers providing that the polymers contain nucleobases in a configuration which allows for base pairing and base stacking, such as is found in DNA and RNA. The term nucleic acid also encompasses linked nucleic acids (LNAs), which are described in U.S. Pat. Nos. 6,794,499, 6,670,461, 6,262,490, and 6,770,748, which are incorporated herein by reference in their entirety for their disclosure of LNAs.

[0047] The nucleic acid(s) can be derived from a completely chemical synthesis process, such as a solid phase-mediated chemical synthesis, from a biological source, such as through isolation from any species that produces nucleic acid, or from processes that involve the manipulation of nucleic acids by molecular biology tools, such as DNA replication, PCR amplification, reverse transcription, or from a combination of those processes.

[0048] The term "target nucleic acids" is used herein to refer to particular nucleic acids to be detected in the methods of the invention.

[0049] As used herein the term "target nucleotide sequence" refers to a molecule that includes the nucleotide sequence of a target nucleic acid, such as, for example, the amplification product obtained by amplifying a target nucleic acid or the cDNA produced upon reverse transcription of an RNA target nucleic acid.

[0050] As used herein, the term "complementary" refers to the capacity for precise pairing between two nucleotides. I.e., if a nucleotide at a given position of a nucleic acid is capable of hydrogen bonding with a nucleotide of another nucleic acid, then the two nucleic acids are considered to be complementary to one another at that position. Complementarity between two single-stranded nucleic acid molecules may be "partial," in which only some of the nucleotides bind, or it may be complete when total complementarity exists between the single-stranded molecules. The degree of complementarity between nucleic acid strands has significant effects on the efficiency and strength of hybridization between nucleic acid strands.

[0051] "Specific hybridization" refers to the binding of a nucleic acid to a target nucleotide sequence in the absence of substantial binding to other nucleotide sequences present in the hybridization mixture under defined stringency conditions. Those of skill in the art recognize that relaxing the stringency of the hybridization conditions allows sequence mismatches to be tolerated.

[0052] In particular embodiments, hybridizations are carried out under stringent hybridization conditions. The phrase "stringent hybridization conditions" generally refers to a temperature in a range from about 5° C. to about 20° C. or 25° C. below than the melting temperature (T_m) for a specific sequence at a defined ionic strength and pH. As used herein, the T_m is the temperature at which a population of double-stranded nucleic acid molecules becomes half-dissociated into single strands. Methods for calculating the T_m of nucleic acids are well known in the art (see, e.g., Berger and Kimmel (1987) METHODS IN ENZYMOLOGY, VOL. 152: GUIDE TO MOLECULAR CLONING TECHNIQUES, San Diego: Academic Press, Inc. and Sambrook et al. (1989) MOLECULAR CLONING: A LABORATORY MANUAL, 2ND ED., VOLS. 1-3, Cold Spring Harbor Laboratory), both incorporated herein by reference). As indicated by standard references, a simple estimate of the T_m value may be calculated by the equation: $T_m = 81.5 + 0.41(\% G+C)$, when a nucleic acid is in aqueous solution at 1 M NaCl (see, e.g., Anderson and Young, Quantitative Filter Hybridization in NUCLEIC ACID HYBRIDIZATION (1985)). The melting temperature of a hybrid (and thus the conditions for stringent hybridization) is affected by various factors such as the length and nature (DNA, RNA, base composition) of the primer or probe and nature of the target nucleic acid (DNA, RNA, base composition, present in solution or immobilized, and the like), as well as the concentration of salts and other components (e.g., the presence or absence of formamide, dextran sulfate, polyethylene glycol). The effects of these factors are well known and are discussed in standard references in the art. Exemplary stringent conditions suitable for achieving specific hybridization of most sequences are: a temperature of at least about 60° C. and a salt concentration of about 0.2 molar at pH7.

[0053] The term "oligonucleotide" is used to refer to a nucleic acid that is relatively short, generally shorter than 200 nucleotides, more particularly, shorter than 100 nucleotides, most particularly, shorter than 50 nucleotides. Typically, oligonucleotides are single-stranded DNA molecules.

[0054] The term "primer" refers to an oligonucleotide that is capable of hybridizing (also termed "annealing") with a nucleic acid and serving as an initiation site for nucleotide (RNA or DNA) polymerization under appropriate conditions (i.e., in the presence of four different nucleoside triphosphates and an agent for polymerization, such as DNA or RNA polymerase or reverse transcriptase) in an appropriate buffer and at a suitable temperature. The appropriate length of a primer depends on the intended use of the primer, but primers are typically at least 7 nucleotides long and, more typically range from 10 to 30 nucleotides, or even more typically from 15 to 30 nucleotides, in length. Other primers can be somewhat longer, e.g., 30 to 50 nucleotides long. In this context, "primer length" refers to the portion of an oligonucleotide or nucleic acid that hybridizes to a complementary "target" sequence and primes nucleotide synthesis. Short primer molecules generally require cooler temperatures to form sufficiently stable hybrid complexes with the template. A primer need not reflect the exact sequence of the template but must be

sufficiently complementary to hybridize with a template. The term “primer site” or “primer binding site” refers to the segment of the target nucleic acid to which a primer hybridizes.

[0055] A primer is said to anneal to another nucleic acid if the primer, or a portion thereof, hybridizes to a nucleotide sequence within the nucleic acid. The statement that a primer hybridizes to a particular nucleotide sequence is not intended to imply that the primer hybridizes either completely or exclusively to that nucleotide sequence. For example, in certain embodiments, amplification primers used herein are said to “anneal to a sample-specific nucleotide tag.” This description encompasses primers that anneal wholly to the nucleotide tag, as well as primers that anneal partially to the nucleotide tag and partially to an adjacent nucleotide sequence, e.g., a target nucleotide sequence. Such hybrid primers can increase the specificity of the amplification reaction.

[0056] The term “primer pair” refers to a set of primers including a 5′ “upstream primer” or “forward primer” that hybridizes with the complement of the 5′ end of the DNA sequence to be amplified and a 3′ “downstream primer” or “reverse primer” that hybridizes with the 3′ end of the sequence to be amplified. As will be recognized by those of skill in the art, the terms “upstream” and “downstream” or “forward” and “reverse” are not intended to be limiting, but rather provide illustrative orientation in particular embodiments.

[0057] A primer pair is said to be “unique” if it can be employed to specifically amplify a particular target nucleotide sequence in a given amplification mixture.

[0058] A second primer pair is “nested” relative to a first primer pair if the first primer pair is employed to amplify a first amplification product and then the second primer pair is employed to amplify a target nucleotide sequence within the first amplification product. Nesting can be used increase the specificity of the amplification reaction.

[0059] A “probe” is a nucleic acid capable of binding to a target nucleic acid of complementary sequence through one or more types of chemical bonds, generally through complementary base pairing, usually through hydrogen bond formation, thus forming a duplex structure. The probe binds or hybridizes to a “probe binding site.” The probe can be labeled with a detectable label to permit facile detection of the probe, particularly once the probe has hybridized to its complementary target. Alternatively, however, the probe may be unlabeled, but may be detectable by specific binding with a ligand that is labeled, either directly or indirectly. Probes can vary significantly in size. Generally, probes are at least 7 to 15 nucleotides in length. Other probes are at least 20, 30, or 40 nucleotides long. Still other probes are somewhat longer, being at least 50, 60, 70, 80, or 90 nucleotides long. Yet other probes are longer still, and are at least 100, 150, 200 or more nucleotides long. Probes can also be of any length that is within any range bounded by any of the above values (e.g., 15-20 nucleotides in length).

[0060] The primer or probe can be perfectly complementary to the target nucleic acid sequence or can be less than perfectly complementary. In certain embodiments, the primer has at least 65% identity to the complement of the target nucleic acid sequence over a sequence of at least 7 nucleotides, more typically over a sequence in the range of 10-30 nucleotides, and often over a sequence of at least 14-25 nucleotides, and more often has at least 75% identity, at least 85% identity, at least 90% identity, or at least 95%, 96%, 97%, 98%, or 99% identity. It will be understood that certain bases

(e.g., the 3′ base of a primer) are generally desirably perfectly complementary to corresponding bases of the target nucleic acid sequence. Primer and probes typically anneal to the target sequence under stringent hybridization conditions.

[0061] The term “nucleotide tag” is used herein to refer to a predetermined nucleotide sequence that is added to a target nucleotide sequence. The nucleotide tag can encode an item of information about the target nucleotide sequence, such as the identity of the target nucleotide sequence, the chromosome from which that sequence derives, or the identity of the sample from which the target nucleotide sequence was derived. In certain embodiments, such information may be encoded in one or more nucleotide tags, e.g., a combination of two nucleotide tags, one on either end of a target nucleotide sequence, can encode the identity of the target nucleotide sequence.

[0062] As used herein, the term “encoding reaction” refers to reaction in which at least one nucleotide tag is added to a target nucleotide sequence. Nucleotide tags can be added, for example, by an “encoding PCR” in which the at least one primer comprises a target-specific portion and a nucleotide tag located on the 5′ end of the target-specific portion, and a second primer that comprises only a target-specific portion or a target-specific portion and a nucleotide tag located on the 5′ end of the target-specific portion. For illustrative examples of PCR protocols applicable to encoding PCR, see pending WO Application US03/37808 as well as U.S. Pat. No. 6,605,451. Nucleotide tags can also be added by an “encoding ligation” reaction that can comprise a ligation reaction in which at least one primer comprises a target-specific portion and nucleotide tag located on the 5′ end of the target-specific portion, and a second primer that comprises a target-specific portion only or a target-specific portion and a nucleotide tag located on the 5′ end of the target specific portion. Illustrative encoding ligation reactions are described, for example, in U.S. Patent Publication No. 2005/0260640, which is hereby incorporated by reference in its entirety, and in particular for ligation reactions. An encoding reaction, such as encoding PCR, can be carried out in 1 cycle, which is sufficient to add a single nucleotide tag. Alternatively, an encoding reaction, such as encoding PCR, can be carried out for multiple cycles to preamplify target nucleic acids (e.g., to increase concentration).

[0063] As used herein an “encoding reaction” produces a “tagged target nucleotide sequence,” which includes a nucleotide tag linked to a target nucleotide sequence.

[0064] The term “sample-specific” nucleotide tag is used herein to refer to a nucleotide tag that encodes the identity of the sample of the target nucleotide sequence to which the tag is, or becomes, linked in an encoding reaction.

[0065] As used herein with reference to a portion of a primer, the term “target-specific nucleotide sequence” refers to a sequence that can specifically anneal to a target nucleic acid or a target nucleotide sequence under suitable annealing conditions.

[0066] A “common” sample-specific nucleotide tag refers to a tag having a specific nucleotide sequence that is, or becomes, linked to all target nucleotide sequences produced during an encoding reaction, such that all tagged target nucleotide sequences produced from a given sample are each identified by a tag having the same sequence.

[0067] A “set-specific nucleotide tag” is a tag that is, or becomes, linked to a plurality of target nucleotide sequences. In certain embodiments of the invention, an assay mixture can

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comprise multiple tagged target nucleotide sequence sequences having different set-specific nucleotide tags at either end that, in combination, uniquely identify each tagged target nucleotide sequence.

[0068] The phrase “a distinct set of forward and reverse primers” refers to a set of primers that is distinguishable from any other sets of primers employed in an assay. Such a set of primers can be used to introduce sample-specific nucleotide tags.

[0069] Amplification according to the present teachings encompasses any means by which at least a part of at least one target nucleic acid is reproduced, typically in a template-dependent manner, including without limitation, a broad range of techniques for amplifying nucleic acid sequences, either linearly or exponentially. Exemplary means for performing an amplifying step include ligase chain reaction (LCR), ligase detection reaction (LDR), ligation followed by Q-replicase amplification, PCR, primer extension, strand displacement amplification (SDA), hyperbranched strand displacement amplification, multiple displacement amplification (MDA), nucleic acid strand-based amplification (NASBA), two-step multiplexed amplifications, rolling circle amplification (RCA), and the like, including multiplex versions and combinations thereof, for example but not limited to, OLA/PCR, PCR/OLA, LDR/PCR, PCR/PCR/LDR, PCR/LDR, LCR/PCR, PCR/LCR (also known as combined chain reaction—CCR), digital amplification, and the like. Descriptions of such techniques can be found in, among other sources, Ausbel et al.; PCR Primer: A Laboratory Manual, Diffenbach, Ed., Cold Spring Harbor Press (1995); The Electronic Protocol Book, Chang Bioscience (2002); Msuih et al., J. Clin. Micro. 34:501-07 (1996); The Nucleic Acid Protocols Handbook, R. Rapley, ed., Humana Press, Totowa, N.J. (2002); Abramson et al., Curr Opin Biotechnol. 1993 February; 4(1):41-7, U.S. Pat. No. 6,027,998; U.S. Pat. No. 6,605,451, Barany et al., PCT Publication No. WO 97/31256; Wenz et al., PCT Publication No. WO 01/92579; Day et al., Genomics, 29(1): 152-162 (1995); Ehrlich et al., Science 252:1643-50 (1991); Innis et al., PCR Protocols: A Guide to Methods and Applications, Academic Press (1990); Favis et al., Nature Biotechnology 18:561-64 (2000); and Rabenau et al., Infection 28:97-102 (2000); Belgrader, Barany, and Lubin, Development of a Multiplex Ligation Detection Reaction DNA Typing Assay, Sixth International Symposium on Human Identification, 1995 (available on the world wide web at: promega.com/geneticidproc/ussymp6proc/blegrad.html); LCR Kit Instruction Manual, Cat. #200520, Rev. #050002, Stratagene, 2002; Barany, Proc. Natl. Acad. Sci. USA 88:188-93 (1991); Bi and Sambrook, Nucl. Acids Res. 25:2924-2951 (1997); Zirvi et al., Nucl. Acid Res. 27:e40i-viii (1999); Dean et al., Proc Natl Acad Sci USA 99:5261-66 (2002); Barany and Gelfand, Gene 109:1-11 (1991); Walker et al., Nucl. Acid Res. 20:1691-96 (1992); Polstra et al., BMC Inf. Dis. 2:18-(2002); Lage et al., Genome Res. 2003 February; 13(2):294-307, and Landegren et al., Science 241:1077-80 (1988), Demidov, V., Expert Rev Mol. Diagn. 2002 November; 2(6):542-8., Cook et al., J Microbiol Methods. 2003 May; 53(2):165-74, Schweitzer et al., Curr Opin Biotechnol. 2001 February; 12(1):21-7, U.S. Pat. No. 5,830,711, U.S. Pat. No. 6,027,889, U.S. Pat. No. 5,686,243, PCT Publication No. WO0056927A3, and PCT Publication No. WO9803673A1.

[0070] In some embodiments, amplification comprises at least one cycle of the sequential procedures of: annealing at

least one primer with complementary or substantially complementary sequences in at least one target nucleic acid; synthesizing at least one strand of nucleotides in a template-dependent manner using a polymerase; and denaturing the newly-formed nucleic acid duplex to separate the strands. The cycle may or may not be repeated. Amplification can comprise thermocycling or can be performed isothermally.

[0071] The term “qPCR” is used herein to refer to quantitative real-time polymerase chain reaction (PCR), which is also known as “real-time PCR” or “kinetic polymerase chain reaction.”

[0072] The term “digital amplification” is used herein to refer to an amplification method in which identical (or substantially similar) amplification reactions are run on a nucleic acid sample, such as genomic DNA. Generally, the quantity of nucleic acid subjected to digital amplification is generally selected such that, when distributed into discrete reaction mixtures, each individual amplification reaction is expected to include one or fewer amplifiable nucleic acids. The concentration of any target amplicon (copies/ μ L) is correlated with the number of positive (i.e., amplification product-containing) reaction mixtures. See compending U.S. application Ser. No. 12/170,414, entitled “Method and Apparatus for Determining Copy Number Variation Using Digital PCR,” which is incorporated by reference for all purposes, and, in particular, for analysis of digital PCR results. Also see Dube et al., 2008, “Mathematical Analysis of Copy Number Variation in a DNA Sample Using Digital PCR on a Nanofluidic Device” PLoS ONE 3(8): e2876. Where PCR is used for amplification, digital amplification is termed “digital PCR.”

[0073] A “reagent” refers broadly to any agent used in a reaction, other than the analyte (e.g., nucleic acid being analyzed). Exemplary reagents for a nucleic acid amplification reaction include, but are not limited to, buffer, metal ions, polymerase, reverse transcriptase, primers, template nucleic acid, nucleotides, labels, dyes, nucleases, and the like. Reagents for enzyme reactions include, for example, substrates, cofactors, buffer, metal ions, inhibitors, and activators.

[0074] The term “universal detection probe” is used herein to refer to any probe that identifies the presence of an amplification product, regardless of the identity of the target nucleotide sequence present in the product.

[0075] The term “universal qPCR probe” is used herein to refer to any such probe that identifies the presence of an amplification product during qPCR. In particular embodiments, nucleotide tags according to the invention can comprise a nucleotide sequence to which a detection probe, such as a universal qPCR probe binds. Where a tag is added to both ends of a target nucleotide sequence, each tag can, if desired, include a sequence recognized by a detection probe. The combination of such sequences can encode information about the identity, chromosomal origin, or sample source of the tagged target nucleotide sequence. In other embodiments, one or more amplification primers can comprise a nucleotide sequence to which a detection probe, such as a universal qPCR probe binds. In this manner, one, two, or more probe binding sites can be added to an amplification product during the amplification step of the methods of the invention. Those of skill in the art recognize that the possibility of introducing multiple probe binding sites during preamplification (if carried out) and amplification facilitates multiplex detection, wherein two or more different amplification products can be detected in a given amplification mixture or aliquot thereof.

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[0076] The term “universal detection probe” is also intended to encompass primers labeled with a detectable label (e.g., a fluorescent label), as well as non-sequence-specific probes, such as DNA binding dyes, including double-stranded DNA (dsDNA) dyes, such as SYBR Green.

[0077] The term “target-specific qPCR probe” is used herein to refer to a qPCR probe that identifies the presence of an amplification product during qPCR, based on hybridization of the qPCR probe to a target nucleotide sequence present in the product.

[0078] “Hydrolysis probes” are generally described in U.S. Pat. No. 5,210,015, which is incorporated herein by reference in its entirety for its description of hydrolysis probes. Hydrolysis probes take advantage of the 5'-nuclease activity present in the thermostable Taq polymerase enzyme typically used in the PCR reaction (TAQMAN® probe technology, Applied Biosystems, Foster City Calif.). The hydrolysis probe is labeled with a fluorescent detector dye such as fluorescein, and an acceptor dye or quencher. In general, the fluorescent dye is covalently attached to the 5' end of the probe and the quencher is attached to the 3' end of the probe, and when the probe is intact, the fluorescence of the detector dye is quenched by fluorescence resonance energy transfer (FRET). The probe anneals downstream of one of the primers that defines one end of the target nucleic acid in a PCR reaction. Using the polymerase activity of the Taq enzyme, amplification of the target nucleic acid is directed by one primer that is upstream of the probe and a second primer that is downstream of the probe but anneals to the opposite strand of the target nucleic acid. As the upstream primer is extended, the Taq polymerase reaches the region where the labeled probe is annealed, recognizes the probe-template hybrid as a substrate, and hydrolyzes phosphodiester bonds of the probe. The hydrolysis reaction irrevocably releases the quenching effect of the quencher dye on the reporter dye, thus resulting in increasing detector fluorescence with each successive PCR cycle. In particular, hydrolysis probes suitable for use in the invention can be capable of detecting 8-mer or 9-mer motifs that are common in the human and other genomes and/or transcriptomes and can have a high T_m of about 70° C. enabled by the use of linked nucleic acid (LNA) analogs.

[0079] The term “label,” as used herein, refers to any atom or molecule that can be used to provide a detectable and/or quantifiable signal. In particular, the label can be attached, directly or indirectly, to a nucleic acid or protein. Suitable labels that can be attached to probes include, but are not limited to, radioisotopes, fluorophores, chromophores, mass labels, electron dense particles, magnetic particles, spin labels, molecules that emit chemiluminescence, electrochemically active molecules, enzymes, cofactors, and enzyme substrates.

[0080] The term “dye,” as used herein, generally refers to any organic or inorganic molecule that absorbs electromagnetic radiation at a wavelength greater than or equal 340 nm.

[0081] The term “fluorescent dye,” as used herein, generally refers to any dye that emits electromagnetic radiation of longer wavelength by a fluorescent mechanism upon irradiation by a source of electromagnetic radiation, such as a lamp, a photodiode, or a laser.

[0082] The term “elastomer” has the general meaning used in the art. Thus, for example, Allcock et al. (Contemporary Polymer Chemistry, 2nd Ed.) describes elastomers in general as polymers existing at a temperature between their glass transition temperature and liquefaction temperature. Elastomeric materials exhibit elastic properties because the polymer chains readily undergo torsional motion to permit uncoiling of the backbone chains in response to a force, with the backbone chains recoiling to assume the prior shape in the absence of the force. In general, elastomers deform when force is applied, but then return to their original shape when the force is removed.

[0083] A “polymorphic marker” or “polymorphic site” is a locus at which nucleotide sequence divergence occurs. Exemplary markers have at least two alleles, each occurring at frequency of greater than 1%, and more typically greater than 10% or 20% of a selected population. A polymorphic site may be as small as one base pair. Polymorphic markers include restriction fragment length polymorphism (RFLPs), variable number of tandem repeats (VNTR's), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, deletions, and insertion elements such as Alu. The first identified allelic form is arbitrarily designated as the reference form and other allelic forms are designated as alternative or variant alleles. The allelic form occurring most frequently in a selected population is sometimes referred to as the wildtype form. Diploid organisms may be homozygous or heterozygous for allelic forms. A diallelic polymorphism has two forms. A triallelic polymorphism has three forms.

[0084] A “single nucleotide polymorphism” (SNP) occurs at a polymorphic site occupied by a single nucleotide, which is the site of variation between allelic sequences. The site is usually preceded by and followed by highly conserved sequences of the allele (e.g., sequences that vary in less than 1/100 or 1/1000 members of the populations). A SNP usually arises due to substitution of one nucleotide for another at the polymorphic site. A transition is the replacement of one purine by another purine or one pyrimidine by another pyrimidine. A transversion is the replacement of a purine by a pyrimidine or vice versa. SNPs can also arise from a deletion of a nucleotide or an insertion of a nucleotide relative to a reference allele.

Detection of Multiple Target Nucleic Acids in Multiple Samples

[0085] In General

[0086] In various embodiments, methods are provided for detecting a plurality of target nucleic acids (i.e., T target nucleic acids, where T is an integer greater than one) in a plurality of samples. In certain embodiments, the method entails providing S samples that will be mixed together (i.e., pooled) prior to assay, where S is an integer greater than 1. Each of these samples is separately subjected to an encoding reaction that produces a set of T tagged target nucleotide sequences, wherein each nucleotide tag encodes information about the identity and/or sample source of a particular target nucleic acid. For each of these S samples (i.e., each of the samples to be pooled), the tagged target nucleotide sequences are mixed to form an assay mixture. In this manner, samples can be assayed in batches, so that, if, e.g., 48 samples are to be analyzed, S can be, e.g., 3, which means that 16 assay mixtures can be prepared to detect 16 different targets. The first assay mixture can contain, e.g., tagged target nucleotide sequences from samples 1-3, the second assay mixture can contain, e.g., tagged target nucleotide sequences from samples 4-6, and so on. See Example 1. An amplification step

is then carried out, whereby the tagged target nucleotide sequences are amplified using at least one primer that anneals to at least one nucleotide tag.

[0087] If greater specificity is desired, one or both amplification primers can include one or more nucleotides that are complementary to the target nucleotide(s) adjacent to the nucleotide tag(s), i.e., hybrid amplification primers including target-specific sequences, as well as tag-specific sequences can be employed. In certain embodiments, the target-specific sequences are 3' of the tag-specific sequences in the primer.

[0088] In principle, the methods described herein are applicable to the detection of as many targets in as many samples as desired. However, the assay format will often dictate the total number of detections that can be performed conveniently without multiplexing, for example, by using more than one type of label. As discussed in detail below, assay mixtures can be aliquoted for separate amplification in, for example, a microtiter plate, or, more typically, a matrix-type microfluidic device. In particular embodiments, S (number of samples that are mixed) \times T (number of targets) defines the number of assay mixture aliquots, which can be, e.g., 30, 48, 96, 120, or 192 for analysis on a matrix-type microfluidic device having the corresponding number of isolated reaction chambers. In exemplary embodiments, the product of the total number of samples assayed in a single assay $\times T$ is at least a value selected from the group consisting of 2304, 3600, 4608, and 9216. As those of skill in the art appreciate, additional increases in throughput can be achieved by multiplexing, for example, by using multiple labels to perform multiple detections in a given assay aliquot.

[0089] Target Nucleotide Sequences Tagged with One Sample-Specific Nucleotide Tag

[0090] In particular embodiments, each tagged target nucleotide sequence includes a sample-specific nucleotide tag and a target nucleotide sequence. In such embodiments, the assay mixture, or aliquots thereof, is subjected to amplification using $S \times T$ unique pairs of amplification primers, wherein each amplification primer pair includes:

[0091] a forward or a reverse amplification primer that anneals to a target nucleotide sequence; and

[0092] a reverse or a forward amplification primer, respectively, that anneals to a sample-specific nucleotide tag. Amplification is therefore dependent upon the presence of a tagged target nucleotide sequence that has a particular target nucleotide sequence and a particular sample-specific tag. Thus, the production of an amplification product including these nucleotide sequences indicates the presence and, if quantitative detection is employed, the amount of a particular target nucleic acid in a particular sample.

[0093] The presence or amount of the amplification product of a unique primer pair can be detected using any means capable of detecting the presence of a target nucleotide sequence in combination with a particular sample-specific tag. For ease of detection, an assay mixture can be analyzed by dividing it into up to $S \times T$ amplification mixtures, and separately subjecting each of the amplification mixtures to amplification using a unique pair of amplification primers. In this case, the presence or amount of an amplification product in a particular aliquot indicates the presence or amount of the target nucleic acid corresponding to the target-specific primer in the sample corresponding to the sample-specific nucleotide tag.

[0094] The nucleotide sequence of the sample-specific nucleotide tag encodes sample identity. All tagged target

nucleotide sequences produced from a given sample can be tagged with a common sample-specific nucleotide tag, i.e., one that has the same nucleotide sequence. Alternatively, every target nucleotide sequence in an assay mixture can be tagged with a distinct sample-specific nucleotide tag, i.e., such that each tagged target nucleotide sequence in the assay mixture bears a sample-specific nucleotide tag having a different nucleotide sequence. Thus, if there are three samples that will be pooled for analysis of 16 targets in one assay mixture, tags 1-16 could be employed to identify target nucleic acid sequences from sample 1, tags 17-32 could be employed to identify target nucleic acid sequences from sample 2, and tags 33-48 could be employed to identify target nucleic acid sequences from sample 3. As those of skill in the art appreciate, sets of tags could be employed such that some, but not all, target nucleotide sequences from a given sample share a common tag. Thus, for example, tag 1 could be employed to identify target nucleic acid sequences 1-4 from sample 1, tag 2 could be employed to identify target nucleic acid sequences 5-8 from sample 1, tag 3 could be employed to identify target nucleic acid sequences 9-12 from sample 1, and tag 4 could be employed to identify target nucleic acid sequences 13-16 from sample 1. In this instance, tags 1-4 would identify sample 1 target nucleic acid sequences, and similar sets of tags (e.g., tags 5-8 and tags 9-12) would identify sample 2 and 3 target nucleic acid sequences.

[0095] In exemplary embodiments of this method, the encoding reaction entails separately subjecting each of the S samples to preamplification using a distinct set of forward and reverse preamplification primers for each sample to produce preamplified samples, wherein each preamplification primer set comprises T pairs of forward and reverse preamplification primers, wherein each preamplification primer pair is capable of amplifying a particular target nucleic acid. In addition, all forward preamplification primers or all reverse preamplification primers in a given set comprise a sample-specific nucleotide tag, which can, but need not, be a common sample-specific nucleotide tag. The sample-specific nucleotide tag is generally 5' of the target-specific nucleotide sequence in the primer.

[0096] The preamplified samples for each of the S samples are mixed to form an assay mixture (e.g., one assay mixture for each set of samples to be analyzed together). The assay mixture can then be analyzed as described above. Each forward preamplification primer in a set can include a sample-specific nucleotide tag, in addition to a target-specific nucleotide sequence, and each reverse preamplification primer in the set can include a target-specific nucleotide sequence. Alternatively, each forward preamplification primer in a set can include a target-specific nucleotide sequence, and each reverse preamplification primer in each set can include a sample-specific nucleotide tag, in addition to a target-specific nucleotide sequence.

[0097] Target Nucleotide Sequences Tagged with Two Nucleotide Tags

[0098] In particular embodiments, each tagged target nucleotide sequence includes a first nucleotide tag linked to a target nucleotide sequence, which is linked to a second nucleotide tag. In such embodiments, the assay mixture, or aliquots thereof, is subjected to amplification using $S \times T$ unique pairs of amplification primers, wherein each amplification primer pair includes:

[0099] a forward or a reverse amplification primer that anneals to a first nucleotide tag; and

[0100] a reverse or a forward amplification primer, respectively, that anneals to a second nucleotide tag. Amplification is therefore dependent upon the presence of a tagged target nucleotide sequence that has the proper combination of tags.

[0101] The nucleotide sequence of the nucleotide tag can encode sample identity in the various ways described above for embodiments employing a single sample-specific tag. In other words, all tagged target nucleotide sequences produced from a given sample can be tagged with a common sample-specific nucleotide tag. Alternatively, every target nucleotide sequence in an assay mixture can be tagged with a distinct sample-specific nucleotide tag, or sets of tags could be employed such that some, but not all, target nucleotide sequences from a given sample share a common tag. Because two tags are used, any of these strategies can be employed in combination. For example, one tag can be a sample-specific tag common to all tagged target nucleotide sequences produced from a given sample, whereas the other tag can be distinct for every tagged nucleotide sequence in an assay mixture. In certain high-specificity embodiments, every tag in an assay mixture is distinct (different in nucleotide sequence) from every other tag in the mixture.

[0102] In exemplary embodiments of this dual tagging method, the encoding reaction entails separately subjecting each of said S samples to preamplification using a distinct set of forward and reverse preamplification primers for each sample to produce preamplified samples, wherein each preamplification primer set comprises T pairs of forward and reverse preamplification primers (i.e., one for each target), wherein each preamplification primer pair is capable of amplifying a particular target nucleic acid. Additionally, each forward preamplification primer comprises a forward nucleotide tag, and each reverse preamplification primer comprises a reverse nucleotide tag. These tags are generally 5' of the target-specific nucleotide sequence in the primer.

[0103] The preamplified samples for each of the S samples are mixed to form an assay mixture (e.g., one assay mixture for each set of samples to be analyzed together). The assay mixture can then be analyzed by amplification as described generally above. Each amplification primer pair includes:

[0104] a forward amplification primer that anneals to a forward nucleotide tag; and

[0105] a reverse amplification primer that anneals to a reverse nucleotide tag.

Detection of Multiple Target Nucleic Acids Through Combinatorial Tagging

[0106] The invention also provides a dual tagging assay method that is useful for detecting a plurality of target nucleic acids in a sample. This method entails providing T forward preamplification primers to a sample, wherein T is the number of targets to be detected. Each forward preamplification primer includes a different target-specific nucleotide sequence and a set-specific nucleotide tag. The set-specific nucleotide tag is generally 5' of the target-specific nucleotide tag. X different forward set-specific nucleotide tags are employed, and X is an integer that is greater than 1 and less than T. Thus, T/X primers comprise the same forward set-specific nucleotide tag.

[0107] Also provided to the sample are T reverse preamplification primers. Each reverse preamplification primer includes a different target-specific nucleotide sequence and a reverse set-specific nucleotide tag. The set-specific nucleotide tag is generally 5' of the target-specific nucleotide tag. Y

different reverse set-specific nucleotide tags are employed, and Y is an integer that is greater than 1 and less than T. Thus, T/Y primers comprise the same reverse set-specific nucleotide tag.

[0108] The sample is subjected to preamplification to produce an assay mixture, wherein any preamplification product produced for a particular target incorporates a unique combination of forward and reverse set-specific nucleotide tags. An amplification step is then carried out, whereby the tagged target nucleotide sequences are amplified by the proper combination of amplification primers, namely those that anneal to the nucleotide tags present in a particular tagged nucleotide sequence.

[0109] Thus, where X+Y primers are prepared, X×Y targets can be analyzed in a single assay. For example, if X and Y are each 100, only 200 primers need be synthesized to detect 10,000 target nucleic acids.

[0110] In exemplary embodiments of this method, the amplification is carried out by dividing the assay mixture into T amplification mixtures, and separately amplifying each of said amplification mixtures using a unique pair of amplification primers. Each amplification primer pair includes:

[0111] a forward amplification primer that anneals to the forward set-specific nucleotide tag; and

[0112] a reverse amplification primer that anneals to the reverse set-specific nucleotide tag.

For each unique primer pair, the presence or amount of an amplification product in the amplification mixture, or aliquot thereof, is determined. The presence of an amplification product indicates the presence of a particular target nucleic acid in the sample.

[0113] In principle, the methods of the invention are applicable to the detection of as many targets in as many samples as desired. However, the assay format will often dictate the total number of detections that can be performed conveniently without multiplexing, for example, by using more than one type of label. In particular embodiments, assay mixtures are aliquoted for separate amplification in, for example, a microtiter plate, or, more typically, a matrix-type microfluidic device. Current microfluidic device designs lend themselves to assays in which X or Y is at least a value selected from 12, 24, 48, and 96, and where T (the number of targets detected) is at least a value selected from the group consisting of 384, 576, 768, 1152, 2304, 3600, 4608, and 9216. As those of skill in the art appreciate, additional increases in throughput can be achieved by multiplexing, for example, by using multiple labels to perform multiple detections in a given assay aliquot.

[0114] The invention also contemplates combinations of the tagging methods described above.

Detection of Multiple Target Nucleic Acids—Modular Approach

[0115] The invention also provides an assay method for detecting a plurality of target nucleic acids in a sample, wherein the target nucleic acids to be detected are divided into sets or “modules,” each module of target nucleic acids is tagged with the same set of nucleotide tag pairs. Within each module, the sets of tag pairs differ from one another, but same set of tag pairs is used for each module. Detection can then be carried out by amplifying each module with a set of primer pairs that anneals to the set of tag pairs.

[0116] More specifically, in certain embodiments, the method entail dividing a sample into R aliquots, wherein R is

an integer greater than 1 (e.g., 96). Each of the R aliquots can be separately subjected to an encoding reaction that produces a set of T tagged target nucleotide sequences, wherein T is the number of target nucleic acids to be detected in each aliquot, T being an integer greater than one (e.g., 96). Each tagged target nucleotide sequence includes a first nucleotide tag 5' of a target nucleotide sequence, a target nucleotide sequence, and a second nucleotide tag 3' of the target nucleotide sequence. The combination of nucleotide tags in each of said T tagged target nucleotide sequences is unique for every tagged target nucleotide sequence in each aliquot. However, the same set of first and second nucleotide tag combinations is used in the encoding reaction in each of the aliquots. Thus, in certain embodiments, the combination of nucleotide tags in each of said T tagged target nucleotide sequences is present in a tagged target nucleotide sequence in each of the other aliquots, although each tag combination can be attached to a different target nucleotide sequence. Accordingly, in particular embodiments the encoding reaction can produce up to $R \times T$ (e.g., $96 \times 96 = 9216$) different tagged target nucleotide sequences, thus permitting the assay of $R \times T$ (e.g., 9216).

[0117] Detection of the tagged target nucleotide sequences can be carried out by separately subjecting each aliquot to amplification using the same set of T different amplification primer pairs for each aliquot, each primer pair including a first primer that anneals to the first nucleotide tag and a second primer that anneals to the second nucleotide tag in each tagged target nucleotide sequence. The presence of an amplification product corresponding to each unique primer pair in each aliquot indicates the presence of a particular target nucleic acid in the sample.

[0118] In certain embodiments, prior to amplification, each aliquot can be divided into T sub-aliquots. Then, one of a set of T different amplification primer pairs can be combined with each sub-aliquot, and the sub-aliquots can be subjected to separate amplification reactions.

[0119] In illustrative embodiments, the encoding reaction can be a preamplification reaction, which may be carried out on a microfluidic device. To increase target nucleic acid concentration prior to encoding, an optional pre-preamplification reaction can be carried out before the encoding preamplification reaction. The pre-preamplification can be carried out in multiplex. For example, target-specific primers for 9216 different target nucleic acids can be employed in one mixture. This mixture can then be divided into $R=96$ aliquots and each aliquot subjected to an encoding preamplification reaction on a microfluidic device, using $T=96$ different primer pairs that add 96 different nucleotide tag pairs to the target nucleotide sequences in each of the 96 aliquots. To increase specificity, the primers employed for preamplification can be nested relative to primers employed for pre-preamplification.

[0120] After the encoding preamplification reaction, amplification can be carried out in separate chambers of a microfluidic device. For example, each of the 96 aliquots produced upon encoding preamplification can be loaded into individual sample lines of a matrix-type microfluidic device, and each of 96 different tag-specific primer combinations can be loaded into individual assay columns. Each different of the 96 primer combination can amplify a different target nucleic acid in each of the 96 aliquots. The resulting 9216 reaction chambers (sub-aliquots) can then be subjected to amplification, followed by detection of amplification product(s), which can be carried out by any suitable means, including SYBR Green, universal probe library, use of one probe per tag com-

bination (e.g., wherein probe sequences are introduced into nucleotide tags), use of fluorescent primers to add nucleotide tags.

[0121] In particular embodiments, this modular approach is extremely flexible and can be easily expanded by additional targets (e.g., an additional 96 targets). It is thus well-suited for assay panels.

Sample Nucleic Acids

[0122] Preparations of nucleic acids ("samples") can be obtained from biological sources and prepared using conventional methods known in the art. In particular, DNA or RNA useful in the methods described herein can be extracted and/or amplified from any source, including bacteria, protozoa, fungi, viruses, organelles, as well higher organisms such as plants or animals, particularly mammals, and more particularly humans. Suitable nucleic acids can also be obtained from environmental sources (e.g., pond water), from man-made products (e.g., food), from forensic samples, and the like. Nucleic acids can be extracted or amplified from cells, bodily fluids (e.g., blood, a blood fraction, urine, etc.), or tissue samples by any of a variety of standard techniques. Exemplary samples include samples of plasma, serum, spinal fluid, lymph fluid, peritoneal fluid, pleural fluid, oral fluid, and external sections of the skin; samples from the respiratory, intestinal genital, and urinary tracts; samples of tears, saliva, blood cells, stem cells, or tumors. For example, samples of fetal DNA can be obtained from an embryo (e.g., from one or a few embryonic or fetal cells) or from maternal blood. Samples can be obtained from live or dead organisms or from in vitro cultures. Exemplary samples can include single cells, paraffin-embedded tissue samples, and needle biopsies. Nucleic acids useful in the invention can also be derived from one or more nucleic acid libraries, including cDNA, cosmid, YAC, BAC, P1, PAC libraries, and the like.

[0123] Nucleic acids of interest can be isolated using methods well known in the art, with the choice of a specific method depending on the source, the nature of nucleic acid, and similar factors. The sample nucleic acids need not be in pure form, but are typically sufficiently pure to allow the amplification steps of the methods of the invention to be performed. Where the target nucleic acids are RNA, the RNA can be reversed transcribed into cDNA by standard methods known in the art and as described in Sambrook, J., Fritsch, E. F., and Maniatis, T., *Molecular Cloning: A Laboratory Manual*. Cold Spring Harbor Laboratory Press, NY, Vol. 1, 2, 3 (1989), for example. The cDNA can then be analyzed according to the methods of the invention.

Target Nucleic Acids

[0124] Any target nucleic acid that can be tagged in an encoding reaction of the invention (described herein) can be detected using the methods of the invention. In typical embodiments, at least some nucleotide sequence information will be known for the target nucleic acids. For example, if the encoding reaction employed is PCR, sufficient sequence information is generally available for each end of a given target nucleic acid to permit design of suitable amplification primers.

[0125] The targets can include, for example, nucleic acids associated with pathogens, such as viruses, bacteria, protozoa, or fungi; RNAs, e.g., those for which over- or under-expression is indicative of disease, those that are expressed in

a tissue- or developmental-specific manner; or those that are induced by particular stimuli; genomic DNA, which can be analyzed for specific polymorphisms (such as SNPs), alleles, or haplotypes, e.g., in genotyping. Of particular interest are genomic DNAs that are altered (e.g., amplified, deleted, and/or mutated) in genetic diseases or other pathologies; sequences that are associated with desirable or undesirable traits; and/or sequences that uniquely identify an individual (e.g., in forensic or paternity determinations).

Primer Design

[0126] Primers suitable for nucleic acid amplification are sufficiently long to prime the synthesis of extension products in the presence of the agent for polymerization. The exact length and composition of the primer will depend on many factors, including, for example, temperature of the annealing reaction, source and composition of the primer, and where a probe is employed, proximity of the probe annealing site to the primer annealing site and ratio of primer:probe concentration. For example, depending on the complexity of the target nucleic acid sequence, an oligonucleotide primer typically contains in the range of about 15 to about 30 nucleotides, although it may contain more or fewer nucleotides. The primers should be sufficiently complementary to selectively anneal to their respective strands and form stable duplexes. One skilled in the art knows how to select appropriate primer pairs to amplify the target nucleic acid of interest.

[0127] For example, PCR primers can be designed by using any commercially available software or open source software, such as Primer3 (see, e.g., Rozen and Skaletsky (2000) *Meth. Mol. Biol.*, 132: 365-386; www.broad.mit.edu/node/1060, and the like) or by accessing the Roche UPL website. The amplicon sequences are input into the Primer3 program with the UPL probe sequences in brackets to ensure that the Primer3 program will design primers on either side of the bracketed probe sequence.

[0128] Primers may be prepared by any suitable method, including, for example, cloning and restriction of appropriate sequences or direct chemical synthesis by methods such as the phosphotriester method of Narang et al. (1979) *Meth. Enzymol.* 68: 90-99; the phosphodiester method of Brown et al. (1979) *Meth. Enzymol.* 68: 109-151; the diethylphosphoramidite method of Beaucage et al. (1981) *Tetra. Lett.*, 22: 1859-1862; the solid support method of U.S. Pat. No. 4,458,066 and the like, or can be provided from a commercial source.

[0129] Primers may be purified by using a Sephadex column (Amersham Biosciences, Inc., Piscataway, N.J.) or other methods known to those skilled in the art. Primer purification may improve the sensitivity of the methods of the invention.

Microfluidic Devices

[0130] In certain embodiments, any of the methods of the invention can be carried out using a microfluidic device. In illustrative embodiments, a matrix-type microfluidic device is one that allows the simultaneous combination of a plurality of substrate solutions with reagent solutions in separate isolated reaction chambers. It will be recognized, that a substrate solution can comprise one or a plurality of substrates and a reagent solution can comprise one or a plurality of reagents. For example, the microfluidic device can allow the simultaneous pair-wise combination of a plurality of different ampli-

fication primers and samples. In certain embodiments, the device is configured to contain a different combination of primers and samples in each of the different chambers. In various embodiments, the number of separate reaction chambers can be greater than 50, usually greater than 100, more often greater than 500, even more often greater than 1000, and sometimes greater than 5000, or greater than 10,000.

[0131] In particular embodiments, the matrix-type microfluidic device is a Dynamic Array ("DA") microfluidic device. A DA microfluidic device is a matrix-type microfluidic device designed to isolate pair-wise combinations of samples and reagents (e.g., amplification primers, detection probes, etc.) and suited for carrying out qualitative and quantitative PCR reactions including real-time quantitative PCR analysis. In some embodiments, the DA microfluidic device is fabricated, at least in part, from an elastomer. DAs are described in PCT publication WO05107938A2 (Thermal Reaction Device and Method For Using The Same) and US Pat. Publication US20050252773A1, both incorporated herein by reference in their entireties for their descriptions of DAs. DAs may incorporate high-density matrix designs that utilize fluid communication vias between layers of the microfluidic device to weave control lines and fluid lines through the device and between layers. By virtue of fluid lines in multiple layers of an elastomeric block, high density reaction cell arrangements are possible. Alternatively DAs may be designed so that all of the reagent and sample channels are in the same elastomeric layer, with control channels in a different layer.

[0132] U.S. Patent Publication No. 2008/0223721 and PCT Publication No. WO 05/107938A2 describe illustrative matrix-type devices that can be used to practice the methods described herein. FIG. 21 of the latter is reproduced as FIG. 1 and shows an illustrative matrix design having a first elastomeric layer 2110 (1st layer) and a second elastomeric layer 2120 (2d layer) each having fluid channels formed therein. For example, a reagent fluid channel in the first layer 2110 is connected to a reagent fluid channel in the second layer 2120 through a via 2130, while the second layer 2120 also has sample channels therein, the sample channels and the reagent channels terminating in sample and reagent chambers 2180, respectively. The sample and reagent chambers 2180 are in fluid communication with each other through an interface channel 2150 that has an interface valve 2140 associated therewith to control fluid communication between each of the chambers 2180 of a reaction cell 2160. In use, the interface is first closed, then reagent is introduced into the reagent channel from the reagent inlet and sample is introduced into the sample channel through the sample inlet; containment valves 2170 are then closed to isolate each reaction cell 2160 from other reaction cells 2160. Once the reaction cells 2160 are isolated, the interface valve 2140 is opened to cause the sample chamber and the reagent chamber to be in fluid communication with each other so that a desired reaction may take place. It will be apparent from this (and the description in WO 05/107938A2) that the DA may be used for reacting M number of different samples with N number of different reagents.

[0133] Although the DAs described above in WO 05/107938 are well suited for conducting the methods described herein, the invention is not limited to any particular device or design. Any device that partitions a sample and/or allows independent pair-wise combinations of reagents and sample may be used. U.S. Patent Publication No. 20080108063 (which is hereby incorporated by reference it

its entirety) includes a diagram illustrating the 48.48 Dynamic Array, a commercially available device available from Fluidigm Corp. (South San Francisco Calif.). It will be understood that other configurations are possible and contemplated such as, for example, 48×96; 96×96; 30×120; etc.

[0134] In specific embodiments, the microfluidic device can be a Digital Array microfluidic device, which is adapted to perform digital amplification. Such devices can have integrated channels and valves that partition mixtures of sample and reagents into nanolitre volume reaction chambers. In some embodiments, the Digital Array microfluidic device is fabricated, at least in part, from an elastomer. Illustrative Digital Array microfluidic devices are described in copending U.S. Applications owned by Fluidigm, Inc. One illustrative embodiment has 12 input ports corresponding to 12 separate sample inputs to the device. The device can have 12 panels, and each of the 12 panels can contain 765 6 nL reaction chambers with a total volume of 4.59 μL per panel. Microfluidic channels can connect the various reaction chambers on the panels to fluid sources. Pressure can be applied to an accumulator in order to open and close valves connecting the reaction chambers to fluid sources. In illustrative embodiments, 12 inlets can be provided for loading of the sample reagent mixture. 48 inlets can be used to provide a source for reagents, which are supplied to the microfluidic device when pressure is applied to accumulator. Additionally, two or more inlets can be provided to provide hydration to the microfluidic device. Hydration inlets are in fluid communication with the device to facilitate the control of humidity associated with the reaction chambers. As will be understood by one of skill in the art, some elastomeric materials that can be utilized in the fabrication of the device are gas permeable, allowing evaporated gases or vapor from the reaction chambers to pass through the elastomeric material into the surrounding atmosphere. In a particular embodiment, fluid lines located at peripheral portions of the device provide a shield of hydration liquid, for example, a buffer or master mix, at peripheral portions of the microfluidic device surrounding the panels of reaction chambers, thus reducing or preventing evaporation of liquids present in the reaction chambers. Thus, humidity at peripheral portions of the device can be increased by adding a volatile liquid, for example water, to hydration inlets. In a specific embodiment, a first inlet is in fluid communication with the hydration fluid lines surrounding the panels on a first side of the device and the second inlet is in fluid communication with the hydration fluid lines surrounding the panels on the other side of the device.

[0135] While the Digital Array microfluidic devices are well-suited for carrying out the digital amplification methods described herein, one of ordinary skill in the art would recognize many variations and alternatives to these devices. The microfluidic device which is the 12.765 Digital Array commercially available from Fluidigm Corp. (South San Francisco, Calif.), includes 12 panels, each having 765 reaction chambers with a volume of 6 nL per reaction chamber. However, this geometry is not required for digital amplification methods. The geometry of a given Digital Array microfluidic device will depend on the particular application. Additional description related to devices suitable for use in the methods described herein is provided in U.S. Patent Application Publication No. 2005/0252773, incorporated herein by reference for its disclosure of Digital Array microfluidic devices.

[0136] In certain embodiments, the methods described herein can be performed using a microfluidic device that

provides for recovery of reaction products. Such devices are described in detail in copending U.S. Application No. 61/166, 105, filed Apr. 2, 2009, which is hereby incorporated by reference in its entirety and specifically for its description of microfluidic devices that permit reaction product recovery and related methods. For example, a digital PCR method for calibrating DNA samples prior to sequencing can be performed on such devices, permitting recovery of amplification products, which can then serve as templates for DNA sequencing.

[0137] Fabrication methods using elastomeric materials and methods for design of devices and their components have been described in detail in the scientific and patent literature. See, e.g., Unger et al., 2000, *Science* 288:113-16; U.S. Pat. No. 6,960,437 (Nucleic acid amplification utilizing microfluidic devices); U.S. Pat. No. 6,899,137 (Microfabricated elastomeric valve and pump systems); U.S. Pat. No. 6,767, 706 (Integrated active flux microfluidic devices and methods); U.S. Pat. No. 6,752,922 (Microfluidic chromatography); U.S. Pat. No. 6,408,878 (Microfabricated elastomeric valve and pump systems); U.S. Pat. No. 6,645,432 (Microfluidic systems including three-dimensionally arrayed channel networks); U.S. Patent Application Publication Nos. 2004/0115838; 20050072946; 20050000900; 20020127736; 20020109114; 20040115838; 20030138829; 20020164816; 20020127736; and 20020109114; PCT Publication Nos. WO 2005/084191; WO05030822A2; and WO 01/01025; Quake & Scherer, 2000, "From micro to nanofabrication with soft materials" *Science* 290: 1536-40; Unger et al., 2000, "Monolithic microfabricated valves and pumps by multilayer soft lithography" *Science* 288:113-116; Thorsen et al., 2002, "Microfluidic large-scale integration" *Science* 298:580-584; Chou et al., 2000, "Microfabricated Rotary Pump" *Biomedical Microdevices* 3:323-330; Liu et al., 2003, "Solving the "world-to-chip" interface problem with a microfluidic matrix" *Analytical Chemistry* 75, 4718-23; Hong et al, 2004, "A nanoliter-scale nucleic acid processor with parallel architecture" *Nature Biotechnology* 22:435-39.

[0138] U.S. Patent Publication No. 20080223721 and PCT Publication No. WO05107938A2 describe exemplary matrix-type devices that can be used to practice the invention. FIG. 21 of the latter is reproduced as FIG. 1 below. FIG. 1 describes an exemplary matrix design having a first elastomeric layer **2110** (1st layer) and a second elastomeric layer **2120** (2d layer) each having fluid channels formed therein. For example, a reagent fluid channel in the first layer **2110** is connected to a reagent fluid channel in the second layer **2120** through a via **2130**, while the second layer **2120** also has sample channels therein, the sample channels and the reagent channels terminating in sample and reagent chambers **2180**, respectively. The sample and reagent chambers **2180** are in fluid communication with each other through an interface channel **2150** that has an interface valve **2140** associated therewith to control fluid communication between each of the chambers **2180** of a reaction cell **2160**. In use, the interface is first closed, then reagent is introduced into the reagent channel from the reagent inlet and sample is introduced into the sample channel through the sample inlet; containment valves **2170** are then closed to isolate each reaction cell **2160** from other reaction cells **2160**. Once the reaction cells **2160** are isolated, the interface valve **2140** is opened to cause the sample chamber and the reagent chamber to be in fluid communication with each other so that a desired reaction may take place. It will be apparent from this (and the description in

WO05107938A2) that the DA may be used for reacting M number of different samples with N number of different reagents.

[0139] Although the DAs described above in WO05107938 are well suited for conducting the assays of the invention, the invention is not limited to any particular device or design. Any device that partitions a sample, and allows independent pairwise combinations of reagents and sample may be used. U.S. Patent Publication No. 20080108063 (which is hereby incorporated by reference in its entirety) includes a diagram illustrating the 48.48 Dynamic Array, a commercially available device available from Fluidigm Corp. (South San Francisco Calif.). It will be understood that other configurations are possible and contemplated such as, for example, 48×96; 96×96; 30×120; etc.

[0140] According to certain embodiments of the invention, the detection and/or quantification of one or more target nucleic acids from one or more samples may generally be carried out on a microfluidic device by obtaining a sample, optionally pre-amplifying the sample, and distributing aliquots of the pre-amplified sample into reaction chambers of a microfluidic device containing the appropriate buffers, primers, optional probe(s), and enzyme(s), subjecting these mixtures to amplification, and querying the aliquots for the presence of amplified target nucleic acids. The sample aliquots may have a volume of in the range of about 1 picoliter to about 500 nanoliters, in the range of about 100 picoliters to about 20 nanoliters, in the range of about 1 nanoliter to about 20 nanoliters, or in the range of about 5 nanoliters to about 15 nanoliters.

[0141] In certain embodiments, multiplex detection is carried out in individual amplification mixture, e.g., in individual reaction chambers of a matrix-type microfluidic device, which can be used to further increase the number of samples and/or targets that can be analyzed in a single assay or to carry out comparative methods, such as comparative genomic hybridization (CGH)-like analysis of multiple loci.

[0142] In specific embodiments, the assay usually has a dynamic range of at least 3 orders of magnitude, more often at least 4, at least 5, at least 6, at least 7, or at least 8 orders of magnitude.

Quantitative Real-Time PCR and Other Detection and Quantitation Methods

[0143] Any method of detection and/or quantitation of nucleic acids can be used in the invention to detect amplification products. In one embodiment, PCR (polymerase chain reaction) is used to amplify and/or quantitate target nucleic acids. In a variation of this embodiment, digital PCR can be employed. In other embodiments, other amplification systems or detection systems are used, including, e.g., systems described in U.S. Pat. No. 7,118,910 (which is incorporated herein by reference in its entirety for its description of amplification/detection systems) and Invader assays; PE BioSystems). In particular embodiments, real-time quantitation methods are used. For example, “quantitative real-time PCR” methods can be used to determine the quantity of a target nucleic acid present in a sample by measuring the amount of amplification product formed during the amplification process itself.

[0144] Fluorogenic nuclease assays are one specific example of a real-time quantitation method that can be used successfully in the methods described herein. This method of monitoring the formation of amplification product involves

the continuous measurement of PCR product accumulation using a dual-labeled fluorogenic oligonucleotide probe—an approach frequently referred to in the literature as the “TaqMan® method.” See U.S. Pat. No. 5,723,591; Heid et al., 1996, Real-time quantitative PCR Genome Res. 6:986-94, each incorporated herein by reference in their entireties for their descriptions of fluorogenic nuclease assays. It will be appreciated that while “TaqMan® probes” are the most widely used for qPCR, the invention is not limited to use of these probes; any suitable probe can be used.

[0145] Other detection/quantitation methods that can be employed in the present invention include FRET and template extension reactions, molecular beacon detection, Scorpion detection, Invader detection, and padlock probe detection.

[0146] FRET and template extension reactions utilize a primer labeled with one member of a donor/acceptor pair and a nucleotide labeled with the other member of the donor/acceptor pair. Prior to incorporation of the labeled nucleotide into the primer during a template-dependent extension reaction, the donor and acceptor are spaced far enough apart that energy transfer cannot occur. However, if the labeled nucleotide is incorporated into the primer and the spacing is sufficiently close, then energy transfer occurs and can be detected. These methods are particularly useful in conducting single base pair extension reactions in the detection of single nucleotide polymorphisms and are described in U.S. Pat. No. 5,945,283 and PCT Publication WO 97/22719.

[0147] With molecular beacons, a change in conformation of the probe as it hybridizes to a complementary region of the amplified product results in the formation of a detectable signal. The probe itself includes two sections: one section at the 5' end and the other section at the 3' end. These sections flank the section of the probe that anneals to the probe binding site and are complementary to one another. One end section is typically attached to a reporter dye and the other end section is usually attached to a quencher dye. In solution, the two end sections can hybridize with each other to form a hairpin loop. In this conformation, the reporter and quencher dye are in sufficiently close proximity that fluorescence from the reporter dye is effectively quenched by the quencher dye. Hybridized probe, in contrast, results in a linearized conformation in which the extent of quenching is decreased. Thus, by monitoring emission changes for the two dyes, it is possible to indirectly monitor the formation of amplification product. Probes of this type and methods of their use are described further, for example, by Piatek et al., 1998, Nat. Biotechnol. 16:359-63; Tyagi, and Kramer, 1996, Nat. Biotechnology 14:303-308; and Tyagi, et al., 1998, Nat. Biotechnol. 16:49-53 (1998).

[0148] The Scorpion detection method is described, for example, by Thelwell et al. 2000, Nucleic Acids Research, 28:3752-3761 and Solinas et al., 2001, “Duplex Scorpion primers in SNP analysis and FRET applications” Nucleic Acids Research 29:20. Scorpion primers are fluorogenic PCR primers with a probe element attached at the 5'-end via a PCR stopper. They are used in real-time amplicon-specific detection of PCR products in homogeneous solution. Two different formats are possible, the “stem-loop” format and the “duplex” format. In both cases the probing mechanism is intramolecular. The basic elements of Scorpions in all formats are: (i) a PCR primer; (ii) a PCR stopper to prevent PCR read-through of the probe element; (iii) a specific probe sequence; and (iv) a fluorescence detection system containing

at least one fluorophore and quencher. After PCR extension of the Scorpion primer, the resultant amplicon contains a sequence that is complementary to the probe, which is rendered single-stranded during the denaturation stage of each PCR cycle. On cooling, the probe is free to bind to this complementary sequence, producing an increase in fluorescence, as the quencher is no longer in the vicinity of the fluorophore. The PCR stopper prevents undesirable read-through of the probe by Taq DNA polymerase.

[0149] Invader assays (Third Wave Technologies, Madison, Wis.) are used particularly for SNP genotyping and utilize an oligonucleotide, designated the signal probe, that is complementary to the target nucleic acid (DNA or RNA) or polymorphism site. A second oligonucleotide, designated the Invader Oligo, contains the same 5' nucleotide sequence, but the 3' nucleotide sequence contains a nucleotide polymorphism. The Invader Oligo interferes with the binding of the signal probe to the target nucleic acid such that the 5' end of the signal probe forms a "flap" at the nucleotide containing the polymorphism. This complex is recognized by a structure specific endonuclease, called the Cleavase enzyme. Cleavase cleaves the 5' flap of the nucleotides. The released flap binds with a third probe bearing FRET labels, thereby forming another duplex structure recognized by the Cleavase enzyme. This time, the Cleavase enzyme cleaves a fluorophore away from a quencher and produces a fluorescent signal. For SNP genotyping, the signal probe will be designed to hybridize with either the reference (wild type) allele or the variant (mutant) allele. Unlike PCR, there is a linear amplification of signal with no amplification of the nucleic acid. Further details sufficient to guide one of ordinary skill in the art are provided by, for example, Neri, B. P., et al., *Advances in Nucleic Acid and Protein Analysis* 3826:117-125, 2000) and U.S. Pat. No. 6,706,471.

[0150] Padlock probes (PLPs) are long (e.g., about 100 bases) linear oligonucleotides. The sequences at the 3' and 5' ends of the probe are complementary to adjacent sequences in the target nucleic acid. In the central, noncomplementary region of the PLP there is a "tag" sequence that can be used to identify the specific PLP. The tag sequence is flanked by universal priming sites, which allow PCR amplification of the tag. Upon hybridization to the target, the two ends of the PLP oligonucleotide are brought into close proximity and can be joined by enzymatic ligation. The resulting product is a circular probe molecule catenated to the target DNA strand. Any unligated probes (i.e., probes that did not hybridize to a target) are removed by the action of an exonuclease. Hybridization and ligation of a PLP requires that both end segments recognize the target sequence. In this manner, PLPs provide extremely specific target recognition.

[0151] The tag regions of circularized PLPs can then be amplified and resulting amplicons detected. For example, TaqMan® real-time PCR can be carried out to detect and quantitate the amplicon. The presence and amount of amplicon can be correlated with the presence and quantity of target sequence in the sample. For descriptions of PLPs see, e.g., Landegren et al., 2003, Padlock and proximity probes for in situ and array-based analyses: tools for the post-genomic era, *Comparative and Functional Genomics* 4:525-30; Nilsson et al., 2006, Analyzing genes using closing and replicating circles *Trends Biotechnol.* 24:83-8; Nilsson et al., 1994, Padlock probes: circularizing oligonucleotides for localized DNA detection, *Science* 265:2085-8.

[0152] In particular embodiments, fluorophores that can be used as detectable labels for probes include, but are not limited to, rhodamine, cyanine 3 (Cy 3), cyanine 5 (Cy 5), fluorescein, Vic™, Liz™, Tamra™, 5-Fam™, 6-Fam™, and Texas Red (Molecular Probes). (Vic™, Liz™, Tamra™, 5-Fam™, 6-Fam™ are all available from Applied Biosystems, Foster City, Calif.).

[0153] Devices have been developed that can perform a thermal cycling reaction with compositions containing a fluorescent indicator, emit a light beam of a specified wavelength, read the intensity of the fluorescent dye, and display the intensity of fluorescence after each cycle. Devices comprising a thermal cycler, light beam emitter, and a fluorescent signal detector, have been described, e.g., in U.S. Pat. Nos. 5,928,907; 6,015,674; and 6,174,670.

[0154] In some embodiments, each of these functions can be performed by separate devices. For example, if one employs a Q-beta replicase reaction for amplification, the reaction may not take place in a thermal cycler, but could include a light beam emitted at a specific wavelength, detection of the fluorescent signal, and calculation and display of the amount of amplification product.

[0155] In particular embodiments, combined thermal cycling and fluorescence detecting devices can be used for precise quantification of target nucleic acids. In some embodiments, fluorescent signals can be detected and displayed during and/or after one or more thermal cycles, thus permitting monitoring of amplification products as the reactions occur in "real-time." In certain embodiments, one can use the amount of amplification product and number of amplification cycles to calculate how much of the target nucleic acid sequence was in the sample prior to amplification.

[0156] According to some embodiments, one can simply monitor the amount of amplification product after a predetermined number of cycles sufficient to indicate the presence of the target nucleic acid sequence in the sample. One skilled in the art can easily determine, for any given sample type, primer sequence, and reaction condition, how many cycles are sufficient to determine the presence of a given target nucleic acid.

[0157] According to certain embodiments, one can employ an internal standard to quantitate the amplification product indicated by the fluorescent signal. See, e.g., U.S. Pat. No. 5,736,333.

[0158] By acquiring fluorescence over different temperatures, it is possible to follow the extent of hybridization. Moreover, the temperature-dependence of PCR product hybridization can be used for the identification and/or quantification of PCR products. Accordingly, the methods described herein encompass the use of melting curve analysis in detecting and/or quantifying amplicons. Melting curve analysis is well known and is described, for example, in U.S. Pat. Nos. 6,174,670; 6,472,156; and 6,569,627, each of which is hereby incorporated by reference in its entirety, and specifically for its description of the use of melting curve analysis to detect and/or quantify amplification products. In illustrative embodiments, melting curve analysis is carried out using a double-stranded DNA dye, such as SYBR Green, Pico Green (Molecular Probes, Inc., Eugene, Oreg.), ethidium bromide, and the like (see Zhu et al., 1994, *Anal. Chem.* 66:1941-48).

[0159] In various embodiments, employing preamplification, the number of preamplification cycles is sufficient to add

one or more nucleotide tags to the target nucleotide sequences, so that the relative copy numbers of the tagged target nucleotide sequences is substantially representative of the relative copy numbers of the target nucleic acids in the sample. For example, preamplification can be carried out for 2-20 cycles to introduce the sample-specific or set-specific nucleotide tags. In other embodiments, detection is carried out at the end of exponential amplification, i.e., during the "plateau" phase, or endpoint PCR is carried out. In this instance, preamplification will normalize amplicon copy number across targets and across samples. In various embodiments, preamplification and/or amplification can be carried out for about: 2, 4, 10, 15, 20, 25, 30, 35, or 40 cycles or for a number of cycles falling within any range bounded by any of these values.

[0160] If desired, tagged target nucleotide sequences generated as described herein may be analyzed by DNA sequencing. Many current DNA sequencing techniques rely on "sequencing by synthesis." These techniques entail library creation, massively parallel PCR amplification of library molecules, and sequencing. Library creation starts with conversion of sample nucleic acids to appropriately sized fragments, ligation of adaptor sequences onto the ends of the fragments, and selection for molecules properly appended with adaptors. The presence of the adaptor sequences on the ends of the library molecules enables amplification of random-sequence inserts. The above-described methods for tagging nucleotide sequences can be substituted for ligation, to introduce adaptor sequences.

[0161] In particular embodiments, the number of library DNA molecules produced in the massively parallel PCR step is low enough that the chance of two molecules associating with the same substrate, e.g. the same bead (in 454 DNA sequencing) or the same surface patch (in Solexa DNA sequencing) is low, but high enough so that the yield of amplified sequences is sufficient to provide a high throughput in automated sequencing. After suitable adaptor sequences are introduced, as discussed above, digital PCR can be employed to calibrate the number of library DNA molecules prior to sequencing by synthesis.

Labeling Strategies

[0162] Any suitable labeling strategy can be employed in the methods of the invention. Where the assay mixture is aliquoted, and each aliquot is analyzed for presence of a single amplification product, a universal detection probe can be employed in the amplification mixture. In particular embodiments, real-time PCR detection can be carried out using a universal qPCR probe. Suitable universal qPCR probes include double-stranded DNA dyes, such as SYBR Green, Pico Green (Molecular Probes, Inc., Eugene, Oreg.), ethidium bromide, and the like (see Zhu et al., 1994, *Anal. Chem.* 66:1941-48). Suitable universal qPCR probes also include sequence-specific probes that bind to a nucleotide sequence present in all amplification products. Binding sites for such probes can be conveniently introduced into the tagged target nucleotide sequences during preamplification (in embodiments employing preamplification) and/or into amplification products during amplification.

[0163] Alternatively, one or more target-specific qPCR probes (i.e., specific for a target nucleotide sequence to be detected) is employed in the amplification mixtures to detect amplification products. Target-specific probes could be useful, e.g., when only a few target nucleic acids are to be

detected in a large number of samples. For example, if only three targets were to be detected, a target-specific probe with a different fluorescent label for each target could be employed. By judicious choice of labels, analyses can be conducted in which the different labels are excited and/or detected at different wavelengths in a single reaction. See, e.g., *Fluorescence Spectroscopy* (Pesce et al., Eds.) Marcel Dekker, New York, (1971); White et al., *Fluorescence Analysis: A Practical Approach*, Marcel Dekker, New York, (1970); Berlman, *Handbook of Fluorescence Spectra of Aromatic Molecules*, 2nd ed., Academic Press, New York, (1971); Griffiths, *Colour and Constitution of Organic Molecules*, Academic Press, New York, (1976); Indicators (Bishop, Ed.). Pergamon Press, Oxford, 19723; and Haugland, *Handbook of Fluorescent Probes and Research Chemicals*, Molecular Probes, Eugene (1992).

Removal of Undesired Reaction Components

[0164] It will be appreciated that reactions involving complex mixtures of nucleic acids in which a number of reactive steps are employed can result in a variety of unincorporated reaction components, and that removal of such unincorporated reaction components, or reduction of their concentration, by any of a variety of clean-up procedures can improve the efficiency and specificity of subsequently occurring reactions. For example, it may be desirable, in some embodiments, to remove, or reduce the concentration of preamplification primers prior to carrying out the amplification steps described herein.

[0165] In certain embodiments, the concentration of undesired components can be reduced by simple dilution. For example, preamplified samples can be diluted about 2-, 5-, 10-, 50-, 100-, 500-, 1000-, 5000-, or 10,000-fold prior to amplification to improve the specificity of the subsequent amplification step. Those of skill in the art appreciate that the dilution can also fall within a range bounded by any of the above values (e.g., about 100-fold to about 1000-fold).

[0166] In some embodiments, undesired components can be removed by a variety of enzymatic means. Examples of suitable enzymatic means include enzymes that digest single-stranded nucleic acids, such as *E. coli* exonuclease I. Excess dNTPs left over from the amplification reaction can be "removed" by treatment with shrimp alkaline phosphatase (SAP), which removes the phosphate groups from dNTPs. Uracil N-glycosylase (UNG) (AmpErase® from Applied Biosystems, Inc., Foster City, Calif.), can be used to prevent unwanted carry-over primers from an initial amplification reaction in which the primers contained dUTP, instead of dTTP. UNG degrades U-containing primers.

[0167] Alternatively, unreacted primers and dNTPs can be removed by column chromatography. For example, gel filtration over Sephadex can be employed for this purpose.

[0168] In particular embodiments, clean-up includes selective immobilization of nucleic acids. For example, desired nucleic acids can be preferentially immobilized on a solid support. In an exemplary embodiment, photo-biotin is attached to desired nucleic acid, and the resulting biotin-labeled nucleic acids immobilized on a solid support comprising an affinity-moiety binder such as streptavidin. Alternatively, unwanted nucleic acids can be immobilized on a solid support and desired nucleic acids harvested by washing.

Use of Blocking Agents During Amplification

[0169] In certain embodiments, amplification can be carried out in the presence of a blocking agent to increase spe-

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cific amplification of the target nucleic acid. Such an agent can suppress background noise generated during amplification, increase specific amplification of one or more target nucleic acids, and/or improve the quality of amplification (e.g., possibly by improving the efficiency of amplification).

[0170] Blocking agents can be employed in any amplification reaction, for example, where a genomic DNA sample is being preamplified or amplified. Genomic DNA contains repetitive nucleotide sequences to which primers may non-specifically hybridize, which may increase background noise and compete with target nucleic acids for primers. The inclusion of a blocking agent in the amplification reaction mixture increases specific amplification of the target nucleic acid. In various embodiments, the increase in specific amplification can be about 10 percent, about 25 percent, about 50 percent, about 75 percent, about 100 percent, about 150 percent, about 200 percent, about 250 percent, about 300 percent, about 350 percent, about 400 percent, about 450 percent, or about 500 percent of the amplification observed in the absence of blocking agent. Without being bound by a particular theory, it is believed that the blocking may act by hybridizing to repetitive sequences in the genomic DNA sample.

[0171] Blocking agents also find particular utility in multiplex amplification reactions using genomic DNA or other types of nucleic acid samples. In multiplex amplification, the presence of multiple primers in the amplification reaction mixture can increase signal attributable to non-specific hybridization of the multiple primers. The inclusion of a blocking agent may suppress this signal.

[0172] In an illustrative embodiment, a nucleic acid blocking agent, such as tRNA, is employed as a blocking agent in an amplification reaction, such as, e.g., PCR. Other blocking agents can include degenerate oligonucleotide primers, repetitive DNA, BSA, or glycogen.

[0173] The blocking agent should present in an amount to increase specific amplification of the target nucleic acid. In certain embodiments, the blocking agent is present at a concentration in the range of about 0.1 $\mu\text{g}/\mu\text{l}$ to about 40 $\mu\text{g}/\mu\text{l}$. In specific embodiments, the blocking agent concentration can be about 0.1, about 0.2, about 0.3, about 0.4, about 0.5, about 0.6, about 0.7, about 1, about 2, about 3, about 4, about 5, about 6, about 7, about 8, about 9, about 10, about 15, about 20, about 25, about 30, about 35, or about 40 $\mu\text{g}/\mu\text{l}$ of the preamplification or amplification reaction mixture or can be any range having any of these values as endpoints (e.g., about 1 $\mu\text{g}/\mu\text{l}$ to about 5 $\mu\text{g}/\mu\text{l}$). Suitable amounts can be also determined empirically, as shown in Example 4.

[0174] In an illustrative embodiment, tRNA is employed as a blocking agent at a concentration in the range of about 1 $\mu\text{g}/\mu\text{l}$ to about 5 $\mu\text{g}/\mu\text{l}$, e.g., about 2 or 3 $\mu\text{g}/\mu\text{l}$.

Applications

[0175] The methods of the invention are applicable to any technique aimed at detecting the presence or amount of one or more target nucleic acids in a nucleic acid sample. Thus, for example, these methods are applicable to identifying the presence of particular polymorphisms (such as SNPs), alleles, or haplotypes, or chromosomal abnormalities, such as amplifications, deletions, or aneuploidy. The methods may be employed in genotyping, which can be carried out in a number of contexts, including diagnosis of genetic diseases or disorders, pharmacogenomics (personalized medicine), quality control in agriculture (e.g., for seeds or livestock), the study and management of populations of plants or animals

(e.g., in aquaculture or fisheries management or in the determination of population diversity), or paternity or forensic identifications. The methods of the invention can be applied to the identification of sequences indicative of particular conditions or organisms in biological or environmental samples. For example, the methods can be used to identify pathogens, such as viruses, bacteria, and fungi). The methods can also be used to characterize environments or microenvironments, e.g., to characterize the microbial species in the human gut.

[0176] These methods can also be employed to determine DNA or RNA (e.g., mRNA, miRNA) copy number. Determinations of aberrant DNA copy number in genomic DNA is useful, for example, in the diagnosis and/or prognosis of genetic defects and diseases, such as cancer. Determination of RNA "copy number," i.e., expression level is useful for expression monitoring of genes of interest, e.g., in different individuals, tissues, or cells under different conditions (e.g., different external stimuli or disease states) and/or at different developmental stages. Primers can also function as probes.

[0177] In addition, the methods can be employed to prepare nucleic acid samples for further analysis, such as, e.g., DNA sequencing.

[0178] Finally, nucleic acid samples can be tagged as a first step, prior subsequent analysis, to reduce the risk that mislabeling or cross-contamination of samples will compromise the results. For example, any physician's office, laboratory, or hospital could tag samples immediately after collection, and the tags could be confirmed at the time of analysis. Similarly, samples containing nucleic acids collected at a crime scene could be tagged as soon as practicable, to ensure that the samples could not be mislabeled or tampered with. Detection of the tag upon each transfer of the sample from one party to another could be used to establish chain of custody of the sample.

Kits

[0179] Kits according to the invention include one or more reagents useful for practicing one or more assay methods of the invention. A kit generally includes a package with one or more containers holding the reagent(s) (e.g., primers and/or probe(s)), as one or more separate compositions or, optionally, as admixture where the compatibility of the reagents will allow. The kit can also include other material(s) that may be desirable from a user standpoint, such as a buffer(s), a diluent (s), a standard(s), and/or any other material useful in sample processing, washing, or conducting any other step of the assay.

[0180] Kits according to the invention generally include instructions for carrying out one or more of the methods of the invention. Instructions included in kits of the invention can be affixed to packaging material or can be included as a package insert. While the instructions are typically written or printed materials they are not limited to such. Any medium capable of storing such instructions and communicating them to an end user is contemplated by this invention. Such media include, but are not limited to, electronic storage media (e.g., magnetic discs, tapes, cartridges, chips), optical media (e.g., CD ROM), RF tags, and the like. As used herein, the term "instructions" can include the address of an internet site that provides the instructions.

[0181] It is understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be sug-

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gested to persons skilled in the art and are to be included within the spirit and purview of this application and scope of the appended claims.

[0182] In addition, all other publications, patents, and patent applications cited herein are hereby incorporated by reference in their entirety for all purposes.

EXAMPLES

Example 1

“More Samples” Chemistry Using Unique Tags for Every Primer

[0183] This example provides an exemplary protocol for carrying out an assay method of the invention to measure gene expression of 16 targets in up to 144 samples using a 48.48 Dynamic Array available from Fluidigm Corporation, South San Francisco, Calif.

[0184] To increase the sample throughput for gene expression using a dynamic array without chip design change, we developed a new protocol to measure gene expression in more than 48 samples on less than 48 (i.e. 16) targets using one M48 chip. This protocol involved tagging, combining and real-time PCR steps. For proof of concept only 3 target genes were encoded and analyzed.

[0185] During the Tagging step, cDNA samples were divided into 3 separate groups. The forward and reverse primers of 3 targets (GAPDH, LDH1, HPRT) for the three groups were synthesized with unique tag sequences appended to the 5' end of the primers (total of 18 different tags) respectively. In this way, each pair of tags defines a specific target amplified and tagged with reagents for a specific group.

[0186] Each group-specific set of tagged primers were pooled together with 33 other, non-tagged gene expression mixes to simulate high complexity in the preamplification.

[0187] In the tagging step (preamplification of targets with the tagged primers), the 3 separate groups c DNA samples (Standard cDNA, eight titrations 4× diluted, from 5 ng to 0.3 pg) were amplified in a 5 µl reaction containing 2.5 µl of 2× Preamp master mix (Applied Biosystems), 1 µl of the multiplex tagging primer mix (one separate mix per sample group), 1 µl cDNA and 0.5 µl DNA-free water. PCR was performed with an initial 15 min at 95° C., followed by 14 cycles of a 2-step amplification profile of 15 sec at 95° C. for denaturation, 4 min at 60° C. for annealing and extension. The PCR products were diluted 100-fold with DNA suspension buffer (low EDTA TE from TEKnova). Then the PCR products from the 3 groups were combined, with every combined sample having one sample from each of the 3 tagging groups. As a comparison, samples from different tag groups were analyzed individually by quantitative real-time PCR. Also, the analysis was performed in parallel with untagged primers and without mixing samples.

[0188] The combined samples were analyzed by real-time quantitative PCR on a dynamic array chip. The 10× assay mix was prepared for each tag pair with the corresponding TaqMan probes in a 5 µl reaction containing 2 µM Taqman probe, 9 µM of two tag-specific primers and 1× assay loading reagent (Fluidigm). A 5 µL sample mix was prepared for each 2.5 µL of (combined or individual) samples, containing 1× TaqMan Universal Master Mix (Applied Biosystems, Foster City, Calif.), 1×DA Sample Loading Reagent. A standard TaqMan gene expression was performed as mentioned above on the dynamic array chip.

[0189] The $\Delta\Delta C_T$ for different samples of HPRT cDNA and GAPDH cDNA was determined against a reference sample (ABC at relative concentration 0.063, grey background). Samples from three groups A, B and C were not mixed (“individual”), mixed with samples of the same concentration (“ABC”) or mixed with samples of different concentration (“A fixed”). Relative concentration refers to the amount of cDNA added to the tagging reaction (“1.000”=5 nano gram). $\Delta\Delta C_T$ values are listed for samples being analyzed with group A, B and C specific tag primers respectively and with untagged primers, i.e. the primers with the sequence of the target specific portion of the pre-amplification primers. The results are shown in Table 1.

TABLE 1

Sample mix	rConc	A-tag-primers	B-tag-primers	C-tag-primers	Untagged primers
individual	0.016	-0.1	-0.2	-0.4	0.1
individual	0.063	-0.1	-0.9	0.1	0.3
individual	0.250	0.6	-0.5	-0.1	0.1
individual	1.000	0.1	-0.5	-0.2	0.1
ABC	0.016	-0.72	-0.79	-0.41	0.93
ABC	0.063	0.0	0.0	0.0	0.0
ABC	0.250	0.2	-0.6	0.0	0.2
ABC	1.000	0.2	-0.4	-0.2	0.0
A fixed	0.016	0.7	-0.5	0.5	0.5
A fixed	0.063	0.4	-0.8	0.1	0.2
A fixed	0.250	0.1	-0.5	-0.3	-0.2
A fixed	1.000	0.7	-0.6	-0.2	0.0
AVG	0.2	-0.5	-0.1	0.2	0.2
STDEV	0.39	0.24	0.24	0.24	0.29

Example 2

“More Samples” Chemistry for Genotyping

[0190] This example provides an exemplary protocol for carrying out an assay method of the invention to genotype 16 single nucleotide polymorphisms (“SNPs”) in 144 samples (including no template controls (“NTCs”)) using a 48.48 Dynamic Array available from Fluidigm Corporation, South San Francisco, Calif.

[0191] To further increase the sample throughput for genotyping using a dynamic array without chip design change, we developed a new protocol to genotype more than 48 samples on less than 48 (i.e. 16) SNPs using one 48.48CS chip. This protocol involved tagging, combining, and genotyping steps (see FIGS. 4, 5, and 6). During the tagging step, DNA samples were divided into 3 separate groups with 47 individuals each (plus one space for NTC). Three sets of 16 5' forward primers for amplifying 16 SNPs were synthesized. Each set had a different Tag sequence appended to the 5' end of the primers respectively. Each 16 forward primers with the same Tag attached were pooled together and mixed with the 16 SNP specific 3' reverse primers to make multiplex tagging primer mix, one for each Tag.

[0192] In the tagging step, the 3 separate groups of 47 tomato DNA samples were amplified in a 5 µl reaction containing 2.5 µl of 2× Multiplex PCR Master Mix (Qiagen), 1 µl of the multiplex tagging primer mix (one separate Tag per sample group), 1 µl DNA at 60 ng/µL and 0.5 µl DNA-free water. PCR was performed with an initial 15 min at 95° C., then followed by 14 cycles of a 2-step amplification profile of 15 sec at 95° C. for denaturation, 4 min at 60° C. for annealing and extension. The PCR products were diluted 100-fold with

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DNA suspension buffer (low EDTA TE from TEKnova). Then the 47 PCR products from the 3 groups were combined into one group of 47 samples, with every combined sample having one from each of the 3 Tagging groups.

[0193] The combined 47 samples plus one sample with DNA suspension buffer as negative control ("NTC") were genotyped on dynamic array chip. The 10× assay mix was prepared for each SNP individually in a 5 µl reaction containing 2 µM Taqman probes (FAM and VIC), 9 µM of corresponding SNP specific reverse primers, 2.5×ROX (Invitrogen), 0.25% of Tween 20 and 9 µM of one of the 3 Tag primers (Tag-1 GTACGGTAGCAGAGA CTTGGTCTG, Tag-2 GACTTAATGCTGC TTGAGACTTGC, and Tag-3 GACATCGT ACCTGACTCAT CGCAC). And three 10× assay mixes were made for each SNP, one for each Tag. Total 48 assay mixes were made for the 16 SNPs, in 3 Tag groups. A 5 µL sample mix was prepared for each 2.5 µL of 1:100 diluted combined samples and NTC, containing 1× TaqMan Universal Master Mix (Applied Biosystems, Foster City, Calif.), 1×GT Sample Loading Reagent (Fluidigm PN 85000741), and 0.05 units/µL additional extra Taq-Gold polymerase (Applied Biosystems). A standard TaqMan genotyping was performed as mentioned above on dynamic array chip. The results are shown in FIGS. 2 and 3.

Example 3

"More Samples" Chemistry for qPCR Gene Expression Profiling

[0194] This example provides an exemplary protocol for carrying out an assay method of the invention to quantify the expression of 16 cDNAs in 36 serially diluted samples using a 96.96 Dynamic Array available from Fluidigm Corporation, South San Francisco, Calif.

[0195] To increase the sample throughput for qPCR, gene expression profiling using a dynamic array without involving a change to chip design, we developed a new protocol. That protocol, (FIGS. 4, 5, and 6) permits examination of mRNA expression levels for 16 genes, present at different levels. FIG. 7 shows the results of analyzing 16 genes in a dilution series of a Universal Reference sample (BioChain) used to validate the dynamic range and $\Delta\Delta C_t$ measurements of "More Samples" gene expression protocols. In particular, FIG. 7 displays the data derived using a 96.96 array. This protocol (FIGS. 4, 5, and 6) involved tagging, combining samples, and qPCR. During the tagging step (FIG. 6), sample was divided into 3 separate groups with 48 tag-specific reactions containing differing dilution series. Three sets of 16 forward primers for amplifying 16 cDNAs were synthesized. Each set bears a different tag sequence appended to the 5' end of the forward primers. Each 16 forward primers with the same 5' Tag were pooled together and mixed with 16 gene specific 3' reverse primers to make a multiplex tagging primer mix, one for each Tag. See FIG. 6.

[0196] In the tagging step, the 3 separate groups of 4-fold serial (1-in-4, 1-in-16, and 1-in-64 fold) dilution of 16 cDNA samples were amplified in a 10 µl reaction containing: 5 µl of 2× PreAmp Master Mix (P/N 438-4769; ABI), 2 µl of 5×(250 nM) multiplex tagging primer mix (one separate Tag per sample group), 3 µl of Universal Reference cDNA (BioChain). PCR was performed with an initial 15 min at 95° C., followed by 17 cycles of a 2-step amplification profile of 15

sec at 95° C. for denaturation, 4 min at 60° C. for annealing and extension. Samples were diluted 1-in-2 in water and stored at 4° C. These PCR products were treated with 4 µl of ExoSAP-IT (P/N 7800, USB,). See FIG. 5. The 144 samples were reduced to 48 combined samples. The combined sample contains only 1 member of each tag group 1, 2 and 3.

[0197] The combined samples were subjected to the PCR on dynamic array chip. 6.5 µl sample mix was prepared for each cDNA mixture containing: 3.5 µL of 2× TaqMan Gene Expression Master mix (ABI), 0.35 µL of 20×GE sample loading reagent (Fluidigm) and 2.45 µL of combined sample, as described above. Assay mixes (5 µL) contained: 2.6 µM of 3' nested reverse primer and 2.6 µM hydrolysis probe, 0.25% of Tween 20 and 2 µM of one of the 3 Tag primers (Tag-1 or 2 or 3). Standard Fluidigm gene expression qPCR was performed on 96.96 chips.

Example 4

Use of tRNA in Amplification of Genomic DNA

[0198] Human genomic DNA was preamplified using standard protocols on the GeneAmp PCR system 9700 (Applied Biosystems, CA) in a 25 µl reaction containing 1× PreAmp master mix (Applied Biosystems, CA), 900 nM primers, about 10 ng of DNA sample, and differing amounts of tRNA (transfer ribonucleic acid, from baker's yeast *S. cerevisiae*, Sigma Chemicals, cat no RS636-1ML). Samples were diluted and analyzed by digital PCR on a 12.765 Digital Array commercially available from Fluidigm Corp. (South San Francisco, Calif.). The thermal cycling protocol followed was similar to that reported in Qin J., Jones R C, Ramakrishnan R. (2008) *Studying copy number variations using a nanofluidic platform Nucleic Acids Research*, Vol. 36, No. 18 e116.

[0199] FIGS. 8 and 9 demonstrate that the addition of tRNA increases the intensity of the specific amplification signal, suppresses background, and improves the quality of specific amplification curves. Table 2, below, shows the increase in specific counts with the addition of tRNA.

TABLE 2

Amount of tRNA	Counts*
None	9
2 µg/µl	290
3 µg/µl	275

*Average number of signals per panel of 12.765 Digital Array

[0200] The examples given above are merely illustrative and are not meant to be an exhaustive list of all possible embodiments, applications or modifications of the invention. Thus, various modifications and variations of the described methods and systems of the invention will be apparent to those skilled in the art without departing from the scope and spirit of the invention. Although the invention has been described in connection with specific embodiments, it should be understood that the invention as claimed should not be unduly limited to such specific embodiments. Indeed, various modifications of the described modes for carrying out the invention which are obvious to those skilled in molecular biology or in the relevant fields are intended to be within the scope of the appended claims.

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SEQUENCE LISTING

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<210> SEQ ID NO 1

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Nucleotide Tag

<400> SEQUENCE: 1

gtacggtagc agagacttgg tctg

24

<210> SEQ ID NO 2

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Nucleotide Tag

<400> SEQUENCE: 2

gacttaatgc tgcttgagac ttgc

24

<210> SEQ ID NO 3

<211> LENGTH: 24

<212> TYPE: DNA

<213> ORGANISM: Artificial Sequence

<220> FEATURE:

<223> OTHER INFORMATION: Nucleotide Tag

<400> SEQUENCE: 3

gacatcgtag ctgactcacc gcac

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1. An assay method for detecting a plurality of target nucleic acids in a plurality of samples, the method comprising:

providing S samples that will be mixed together prior to assay, where S is an integer greater than 1;

separately subjecting each of said S samples to an encoding reaction that produces a set of T tagged target nucleotide sequences, each tagged target nucleotide sequence comprising a sample-specific nucleotide tag and a target nucleotide sequence; wherein T is the number of target nucleic acids to be detected, T being an integer greater than one;

for each of said S samples, mixing tagged target nucleotide sequences to form an assay mixture;

subjecting the assay mixture, or aliquots thereof, to amplification using S×T unique pairs of amplification primers, wherein each amplification primer pair comprises: a forward or a reverse amplification primer that anneals to a target nucleotide sequence; and

a reverse or a forward amplification primer, respectively, that anneals to a sample-specific nucleotide tag; and

for each unique primer pair, determining whether an amplification product is present in the amplification mixture, or aliquot thereof, whereby the presence of an amplification product indicates the presence of a particular target nucleic acid in a particular sample.

2. The method of claim 1, wherein:

said encoding reaction comprises separately subjecting each of said S samples to preamplification using a dis-

tinct set of forward and reverse preamplification primers for each sample to produce preamplified samples, wherein

each preamplification primer set comprises T pairs of forward and reverse preamplification primers, wherein each preamplification primer pair is capable of amplifying a particular target nucleic acid; and either all forward preamplification primers or all reverse preamplification primers in a given set comprise a common sample-specific nucleotide tag;

said mixing comprises mixing the preamplified samples for each of said S samples to form an assay mixture;

said amplification comprises dividing the assay mixture into up to S×T amplification mixtures, and separately subjecting each of said amplification mixtures to amplification using a unique pair of amplification primers, wherein each amplification primer pair comprises:

a forward or a reverse amplification primer that anneals to a target nucleotide sequence; and

a reverse or a forward amplification primer, respectively, that anneals to a sample-specific nucleotide tag; and

said determining comprises determining whether an amplification product is present in the amplification mixtures, whereby the presence of an amplification product in an amplification mixture indicates the presence of a particular target nucleic acid in a particular sample.

3. The assay method of claim 2, wherein each forward preamplification primer in a set comprises a common sample-

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specific nucleotide tag, in addition to a target-specific nucleotide sequence, and each reverse preamplification primer in each set comprises a target-specific nucleotide sequence.

4. The assay method of claim 2, wherein each forward preamplification primer in a set comprises a target-specific nucleotide sequence, and each reverse preamplification primer in each set comprises a common sample-specific nucleotide tag, in addition to a target-specific nucleotide sequence.

5. An assay method for detecting a plurality of target nucleic acids in a plurality of samples, the method comprising:

providing S samples that will be mixed together prior to assay, where S is an integer greater than 1;

separately subjecting each of said S samples to an encoding reaction that produces a set of T tagged target nucleotide sequences, each tagged target nucleotide sequence comprising a first nucleotide tag linked to a target nucleotide sequence, which is linked to a second nucleotide tag; wherein T is the number of target nucleic acids to be detected, T being an integer greater than one;

for each of said S samples, mixing tagged target nucleotide sequences to form an assay mixture;

subjecting the assay mixture, or aliquots thereof, to amplification using S×T unique pairs of amplification primers, wherein each amplification primer pair comprises: a forward or a reverse amplification primer that anneals to a first nucleotide tag; and

a reverse or a forward amplification primer, respectively, that anneals to a second nucleotide tag; and

for each unique primer pair, determining whether an amplification product is present in the amplification mixture, or aliquot thereof, whereby the presence of an amplification product indicates the presence of a particular target nucleic acid in a particular sample.

6. The method of claim 5, wherein:

said encoding reaction comprises separately subjecting each of said S samples to preamplification using a distinct set of forward and reverse preamplification primers for each sample to produce preamplified samples, wherein

each preamplification primer set comprises T pairs of forward and reverse preamplification primers, wherein each preamplification primer pair is capable of amplifying a particular target nucleic acid; and

each forward preamplification primer comprises a forward nucleotide tag, and each reverse preamplification primer comprises a reverse nucleotide tag;

said mixing comprises mixing the preamplified samples for each of said S samples to form an assay mixture;

said amplification comprises dividing each assay mixture into up to S×T amplification mixtures, and separately subjecting each of said amplification mixtures to amplification using a unique pair of amplification primers, wherein each amplification primer pair comprises:

a forward amplification primer that anneals to a forward nucleotide tag; and

a reverse amplification primer that anneals to a reverse nucleotide tag; and

said determining comprises determining whether an amplification product is present in the amplification mixtures, whereby the presence of an amplification product in an amplification mixture indicates the presence of a particular target nucleic acid in a particular sample.

7. The method of claim 5, wherein at least one of said nucleotide tags comprises a sample-specific nucleotide tag that is common to all tagged target nucleotide sequences produced from a given sample.

8. The method of claim 7, wherein the other nucleotide tag is distinct for each tagged target nucleotide sequence in the assay mixture.

9. The assay method of claim 1, wherein at least one of said amplification primers, comprises at least one nucleotide that is complementary to the target nucleotide adjacent to at least one of said nucleotide tags.

10. The assay method of claim 1, wherein a series of samples is assayed by preparing a plurality of different assay mixtures, wherein each assay mixture comprises a mixture of S different samples.

11. The assay method of claim 10, wherein S×T is at least a value selected from the group consisting of 30, 48, 96, 120, and 192.

12. The assay method of claim 10, wherein the product of the total number of samples assayed in a single assay ×T is at least a value selected from the group consisting of 2304, 3600, 4608, and 9216.

13. An assay method for detecting a plurality of target nucleic acids in a sample, the method comprising:

providing T forward preamplification primers to a sample, wherein each forward preamplification primer comprises a different target-specific nucleotide sequence and a set-specific nucleotide tag, wherein X different forward set-specific nucleotide tags are employed, wherein T is the number of targets to be detected, and X is an integer that is greater than 1 and less than T, whereby T/X primers comprise the same forward set-specific nucleotide tag;

providing T reverse preamplification primers to a sample, wherein each reverse preamplification primer comprises a different target-specific nucleotide sequence and a reverse set-specific nucleotide tag, wherein Y different reverse set-specific nucleotide tags are employed, and Y is an integer that is greater than 1 and less than T, whereby T/Y primers comprise the same reverse set-specific nucleotide tag;

subjecting the sample to preamplification to produce an assay mixture, wherein any preamplification product produced for a particular target incorporates a unique combination of forward and reverse set-specific nucleotide tags;

subjecting the assay mixture, or aliquots thereof, to amplification using amplification primers wherein each amplification primer pair comprises:

a forward amplification primer that anneals to the forward set-specific nucleotide tag; and

a reverse amplification primer that anneals to the reverse set-specific nucleotide tag; and

for each unique primer pair, determining whether an amplification product is present in the amplification mixture, or aliquot thereof, whereby the presence of an amplification product indicates the presence of a particular target nucleic acid in a particular sample.

14-16. (canceled)

17. An assay method for detecting a plurality of target nucleic acids in a sample, the method comprising:

dividing a sample into R aliquots, wherein R is an integer greater than 1;

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separately subjecting each of said R aliquots to an encoding reaction that produces a set of T tagged target nucleotide sequences, wherein T is the number of target nucleic acids to be detected in each aliquot, T being an integer greater than 1, and wherein:

each tagged target nucleotide sequence comprises a first nucleotide tag 5' of a target nucleotide sequence, a target nucleotide sequence, and a second nucleotide tag 3' of the target nucleotide sequence;

the combination of nucleotide tags in each of said T tagged target nucleotide sequences is unique for every tagged target nucleotide sequence in each aliquot; and the same set of first and second nucleotide tag combinations is used in the encoding reaction in each of the aliquots;

separately subjecting each aliquot to amplification using the same set of T different amplification primer pairs for each aliquot, each primer pair comprising a first primer that anneals to the first nucleotide tag and a second primer that anneals to the second nucleotide tag in each tagged target nucleotide sequence; and

for each unique primer pair in each aliquot, determining whether an amplification product is present in the aliquot, whereby the presence of an amplification product indicates the presence of a particular target nucleic acid in the sample.

18-22. (canceled)

23. The assay method of claim 2, wherein amplification mixtures are formed in or, distributed into, separate compartments of a microfluidic device prior to amplification.

24. The assay method of claim 23, wherein the microfluidic device is fabricated, at least in part, from an elastomeric material.

25. The assay method of claim 23, wherein the assay has a dynamic range of at least 4 orders of magnitude.

26. The assay method of claim 2, wherein the preamplification and/or the amplification is carried out by polymerase chain reaction (PCR).

27. The assay method of claim 2, wherein the preamplification is carried out for 2-20 cycles to introduce the nucleotide tags.

28. The assay method of claim 2, wherein the preamplification is carried out for a sufficient number of cycles to normalize amplicon copy number across targets and across samples.

29. The assay method of claim 2, wherein the presence of an amplification product is determined by quantitative real-time polymerase chain reaction (qPCR).

30. The assay method of claim 2, wherein a universal qPCR probe is employed in the amplification mixtures to detect amplification products.

31. The assay method of claim 2, wherein one or more target-specific qPCR probes is employed in the amplification mixtures to detect amplification products.

32. The assay method of claim 2, wherein one or more tag-specific qPCR probes is employed in the amplification mixtures to detect amplification products.

33. The assay method of claim 2, wherein the presence of an amplification product is detected using a fluorogenic nuclease assay.

34. The assay method of claim 33, wherein the presence of an amplification product is detected using a dual-labeled fluorogenic oligonucleotide probe.

35. The assay method of claim 2, additionally comprising quantifying the amount of amplification product in the amplification mixtures.

36. The assay method of claim 35, additionally comprising determining the amount of each target nucleic acid present in each sample.

37. The assay method of claim 2, wherein the assay is performed to determine the copy numbers of the target nucleic acids.

38. The assay method of claim 2, wherein the assay is performed to determine genotypes at loci corresponding to the target nucleic acids.

39. The assay method of claim 2, wherein the assay is performed to determine the expression levels of the target nucleic acids.

40. The assay method of claim 2, additionally comprising, reducing the concentration of preamplification primers prior to carrying out said amplification.

41. The method of claim 2, wherein the sample comprises a genomic DNA sample.

42. The method of claim 41, wherein the preamplification is conducted in the presence of an amount of a blocking agent that is sufficient to increase specific amplification of the target nucleic acid.

43. The method of claim 42, wherein the blocking agent comprises a nucleic acid blocking agent that hybridizes to repetitive sequences in the genomic DNA sample.


44. The method of claim 42, wherein the blocking agent is selected from the group consisting of tRNA, degenerate oligonucleotide primers, repetitive DNA, bovine serum albumin (BSA), and glycogen.


45. The method of claim 42, wherein the blocking agent is present at a concentration in the range of about 0.1 µg/µl to about 40 µg/µl.


46. The method of claim 45, wherein the blocking agent comprises tRNA at a concentration in the range of about 1 µg/µl to about 5 µg/µl.


* * * * *


Exhibit 14

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Abstract 1164: Parallel preparation of targeted resequencing libraries from 480 genomic regions using multiplex PCR on the Access Array system FREE

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— Author & Article Information

Online ISSN: 1538-7445
Print ISSN: 0008-5472

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Cancer Res (2010) 70 (8_Supplement): 1164.
<https://doi.org/10.1158/1538-7445.AM10-1164>

Abstract

Next generation sequencing platforms have dramatically reduced sequencing costs. However, it currently remains too expensive to routinely resequence entire human genomes in order to discover genetic variants or somatic mutations underlying tumorigenesis. Therefore, a need exists for multiplexed, targeted amplification methods that allow for the analysis of multiple genomic regions in large cohorts. Available targeted enrichment technologies are either aimed at the capture of regions of interest from a single sample, exhibit uneven representation or require significant amounts of input material. The novel microfluidic platform, the Access Array system, combines 48 samples with 48 primer sets resulting in 2,304 simultaneously occurring PCR amplifications requiring as little as 50ng DNA per sample. PCR products generated on the Access Array system can be used for sequencing on all next-generation sequencing platforms, including 454 GS FLX and Illumina GAII. To increase coverage and throughput, PCR reactions can be multiplexed within Access Array chips generating up to 480 amplicons per sample.

As proof-of-principal experiments, we have carried out multiplexed amplifications of a set of commonly mutated cancer gene exons across 48 genomic DNA samples. In initial experiments, 580 primer pairs were validated in individual PCR reactions in 96-well plates. Each primer pair was designed to include 5' sequences that allow for the incorporation of 454 and Illumina adapters necessary for subsequent emPCR and cluster generation, respectively. 480 primer pairs that produced a single band of the correct size, as determined on a Caliper LabChip system, were selected for multiplex PCR experiments. Primer pairs yielding amplicons with a similar size were combined in groups of 10 sets, resulting in 48 primer pools of 10 primer pairs each. Multiplex PCR was carried out on Access Array chips, followed by harvesting of the 48 amplicon pools. Each pool was diluted and then subjected to a second round of PCR in standard 96-well plates with barcoded universal primers corresponding to the 454 and Illumina sequences. The resulting products are 48 uniquely barcoded amplicon pools, each comprising 480 amplicons derived from one sample, that are ready for sequencing. We will present sequencing data generated on both 454 and Illumina systems.

Citation Format: {Authors}. {Abstract title} [abstract]. In: Proceedings of the 101st Annual Meeting of the American Association for Cancer Research; 2010 Apr 17-21; Washington, DC. Philadelphia (PA): AACR; Cancer Res 2010;70(8 Suppl):Abstract nr 1164.

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Exhibit 15

Parallel Preparation of Targeted Resequencing Libraries from 480 Genomic Regions Using Multiplex PCR on the Access Array™ System

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Fluidigm Corporation, 7000 Shoreline Court, Suite 100, South San Francisco, CA 94080

Introduction

Next generation sequencing platforms have dramatically reduced sequencing costs. However, it currently remains too expensive to routinely resequence entire human genomes in order to discover genetic variants or somatic mutations underlying tumorigenesis. Therefore, a need exists for multiplexed, targeted amplification methods that allow for the analysis of multiple genomic regions in large cohorts. Available targeted enrichment technologies are either aimed at the capture of regions of interest from a single sample, exhibit uneven representation or require significant amounts of input material. The novel microfluidic platform, the Access Array™ system, combines 48 samples with 48 primer sets resulting in 2,304 simultaneously occurring PCR amplifications requiring as little as 50ng DNA per sample. PCR products generated on the Access Array system can be used for sequencing on all next-generation sequencing platforms, including 454 GS FLX and Illumina GALLi. To increase coverage and throughput, PCR reactions can be multiplexed within Access Array IFCs generating up to 480 amplicons per sample.

Access Array™ System

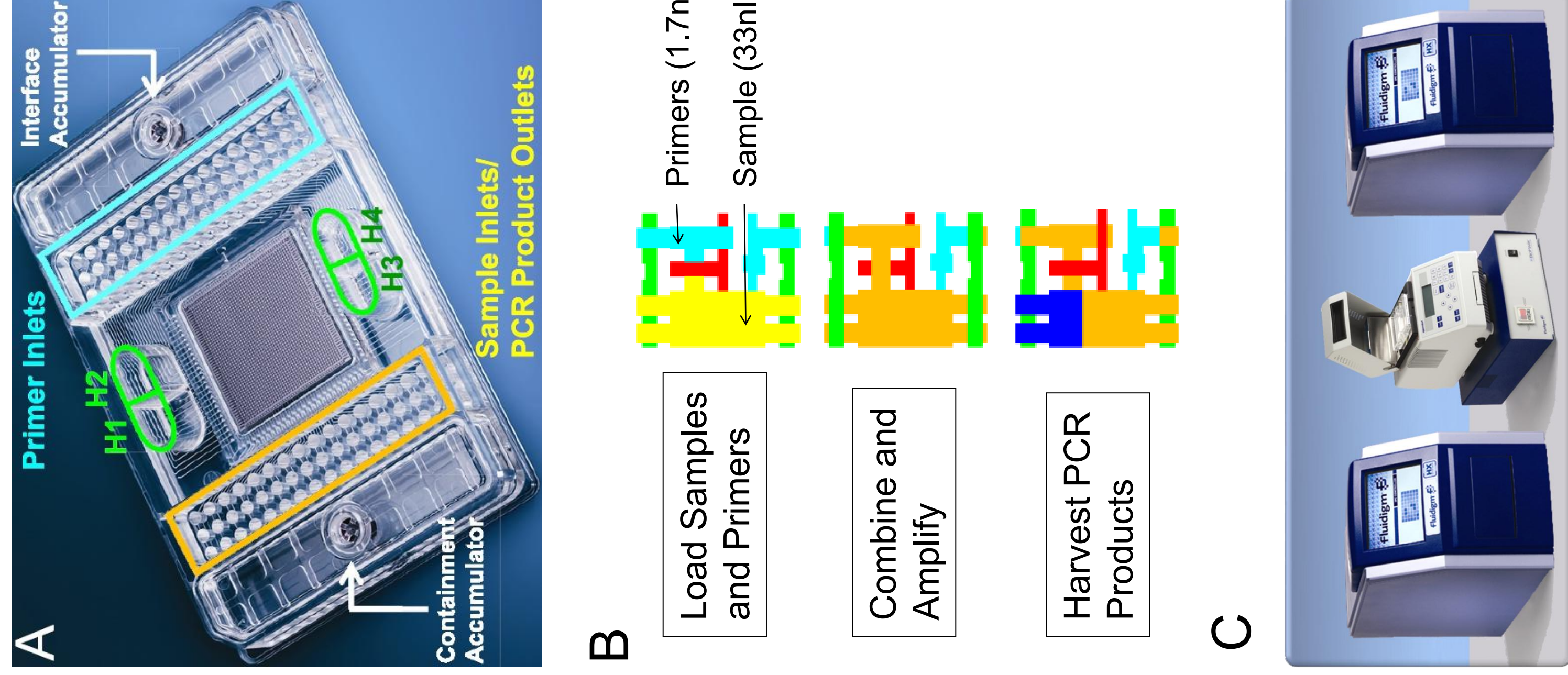
The Access Array system is centered on the 48.48 Access Array Integrated Fluidic Circuit (IFC) (Figure 1A). This is a microfluidic chip that systematically combines 48 sample inputs with 48 primer inputs to make all possible 2304 combinations of samples and primers. The chip is mounted on an SBS-compatible carrier, for simple loading of reagents with an 8-channel pipettor. Once samples and primers have been loaded, and combined into the discrete 2304 reactions, the IFC undergoes thermal cycling to amplify regions of interest from the samples. After thermal cycling has been completed, reaction products are recovered on a per-sample basis using a pump system built into the chip (Figure 1B).

The 48.48 Access Array IFC requires only 5 ul PCR master mix and 50ng input Human genomic DNA per sample. Genomic DNA can be used directly and requires no additional fragmentation or modification before loading into the chip

The Access Array system (Figure 1C) comprises two IFC-controllers, one pre-PCR for loading samples and primers and one post-PCR for harvesting PCR products, and a thermal cycler. When used in conjunction with the Biomark™ real-time PCR system, the progress of individual PCR reactions can be monitored during amplification

When used in combination with primers designed for Amplicon tagging, the Access Array produces sequencer-ready amplicon libraries that can be introduced to emulsion PCR for 454 sequencing. The output from an Access Array IFC is well matched to the capacity of the 454 FLX Sequencer with Titanium chemistry. 48 samples prepared on an Access Array IFC can be sequenced at an average of 50x coverage on 1/8 of a picotiter plate.

Figure 1: The Access Array System



Sequencer-ready PCR products

We have developed a multiplexed amplicon tagging method (Figure 2) that enables direct attachment of sample-specific barcodes and sequencer-specific tags to PCR products generated in the Access Array IFC. Region-specific primers are tagged at the 5' end with universal sequence tags (Figure 2A), 48 pools of up to 10 Region-specific primer pairs are pooled and loaded into the assay inlets of the Access Array IFC. The 48.48 Access Array IFC then combines the region-specific primer pools with the samples in each reaction. After harvesting the PCR product pools from the 48.48 Access Array IFC, a second PCR is carried out on a 96-well plate with barcode primers comprising 454 sequence tags, a barcode sequence, and the universal tags (Figure 2B). The final result is a fully tagged, barcoded PCR product (Figure 2C).

Access Array IFC

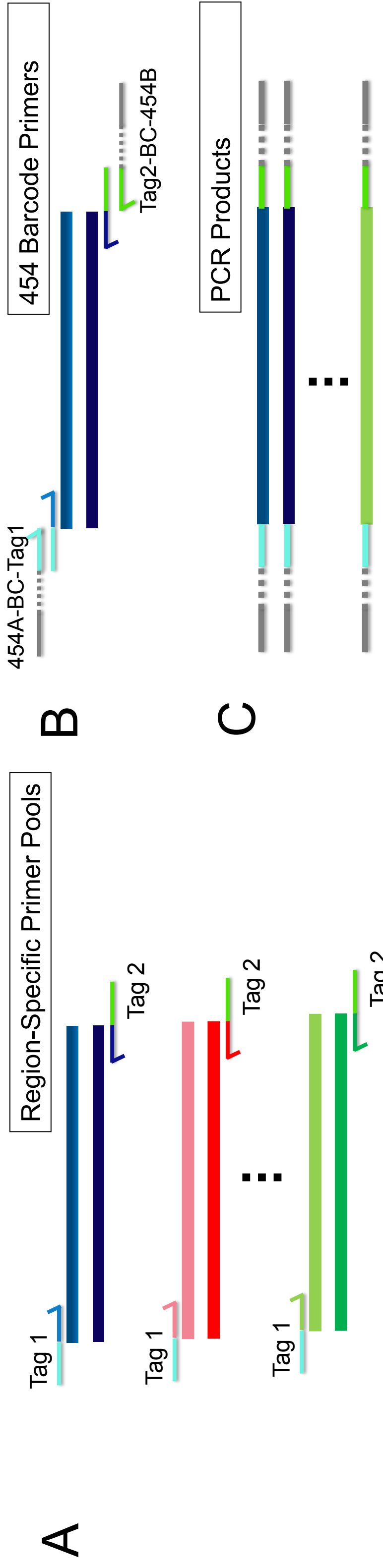


Figure 2: Amplicon tagging to introduce sequencer-specific tags and sample-specific barcodes

High quality sequence data

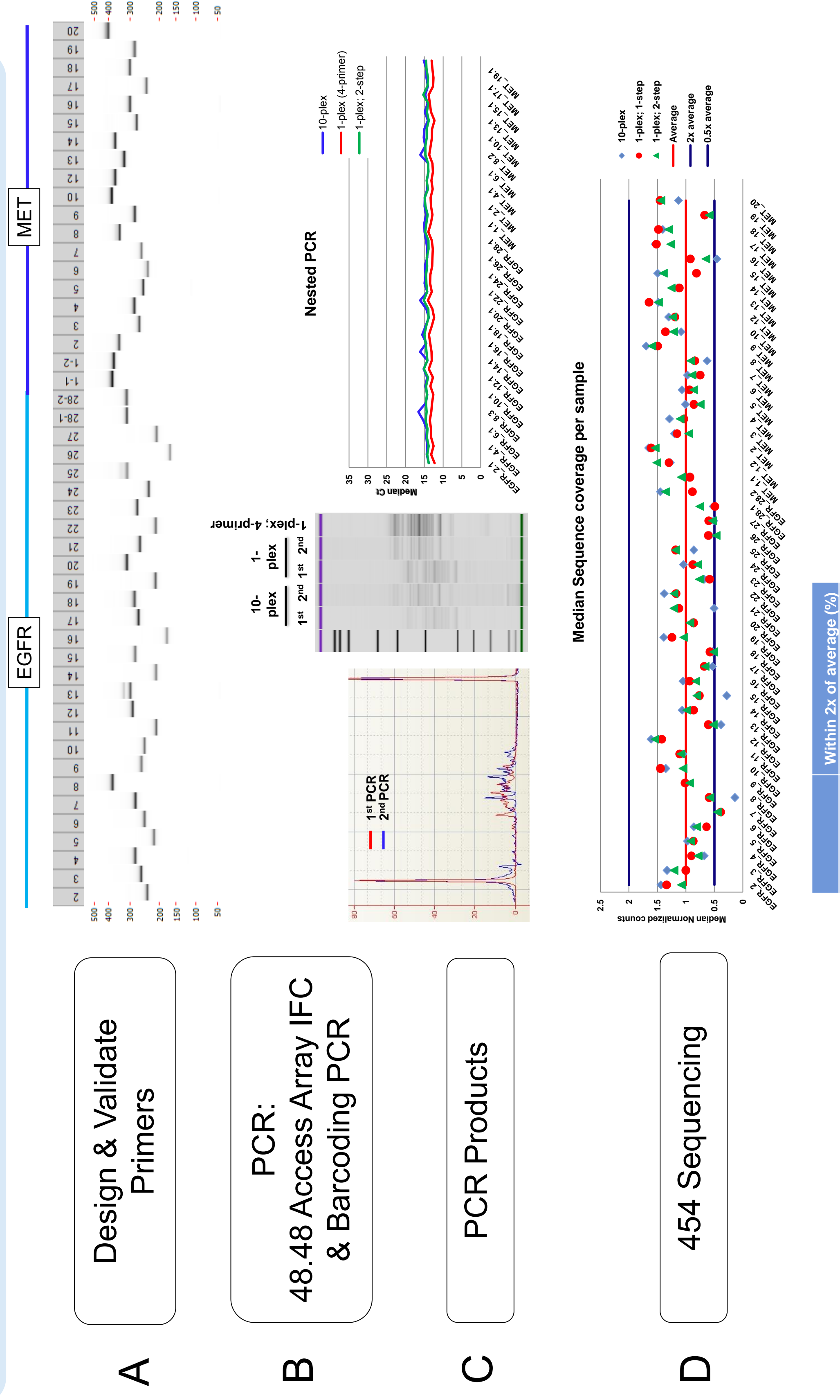


Figure 3: EGFR and MET sequencing

In an initial proof of principle experiment, 48 Primer sets covering the EGFR and MET exons, were designed and validated by PCR (A). The 48 regions were amplified from a HapMap control sample (normalized to 50ng/ul) using 5 pools of 9 to 10 primer pairs each, following the strategy outlined above (Figure 2). The remaining 43 assay inlets on the Access Array IFC were filled with buffer. A comparison was made to singleplex, 2-step PCR and our singleplex, 1-step amplicon tagging strategy. Final PCR products (C) were analyzed on an Agilent BioAnalyzer 2100 and amplicon representation was assessed with a nested Q-PCR. The PCR products were subsequently run on a 454 FLX sequencer. Representation of each PCR product was even, with 90% represented within 2-fold of average coverage (D).

480 Cancer Gene Exons

To maximally utilize the capacity of an 48.48 Access Array IFC, we carried out multiplexed amplifications of a set of commonly mutated cancer gene exons across 48 genomic DNA samples. In initial experiments, 580 tagged primer pairs were validated in individual PCR reactions in 384-well plates. 480 primer pairs that produced a single band of the correct size, as determined on a Caliper LabChip system, were selected for multiplex PCR experiments. Primer pairs were grouped in sets of 10 based on their expected PCR product sizes, resulting in 48 primer pools of 10 primer pairs each. Multiplex PCR was carried out following the strategy outlined in Figure 2. The resulting products are 48 uniquely barcoded PCR product pools, each comprising 480 amplicons derived from one sample, that are ready for sequencing. Harvested PCR product pools were analyzed on an Agilent BioAnalyzer 2100 (Figure 4A) and PCR product representation was assessed with a nested Q-PCR for a subset of amplicons (Figure 4B). The PCR products were subsequently run on a 454 FLX sequencer. Representation of each PCR product was even, with 90% represented within 5-fold of average coverage.

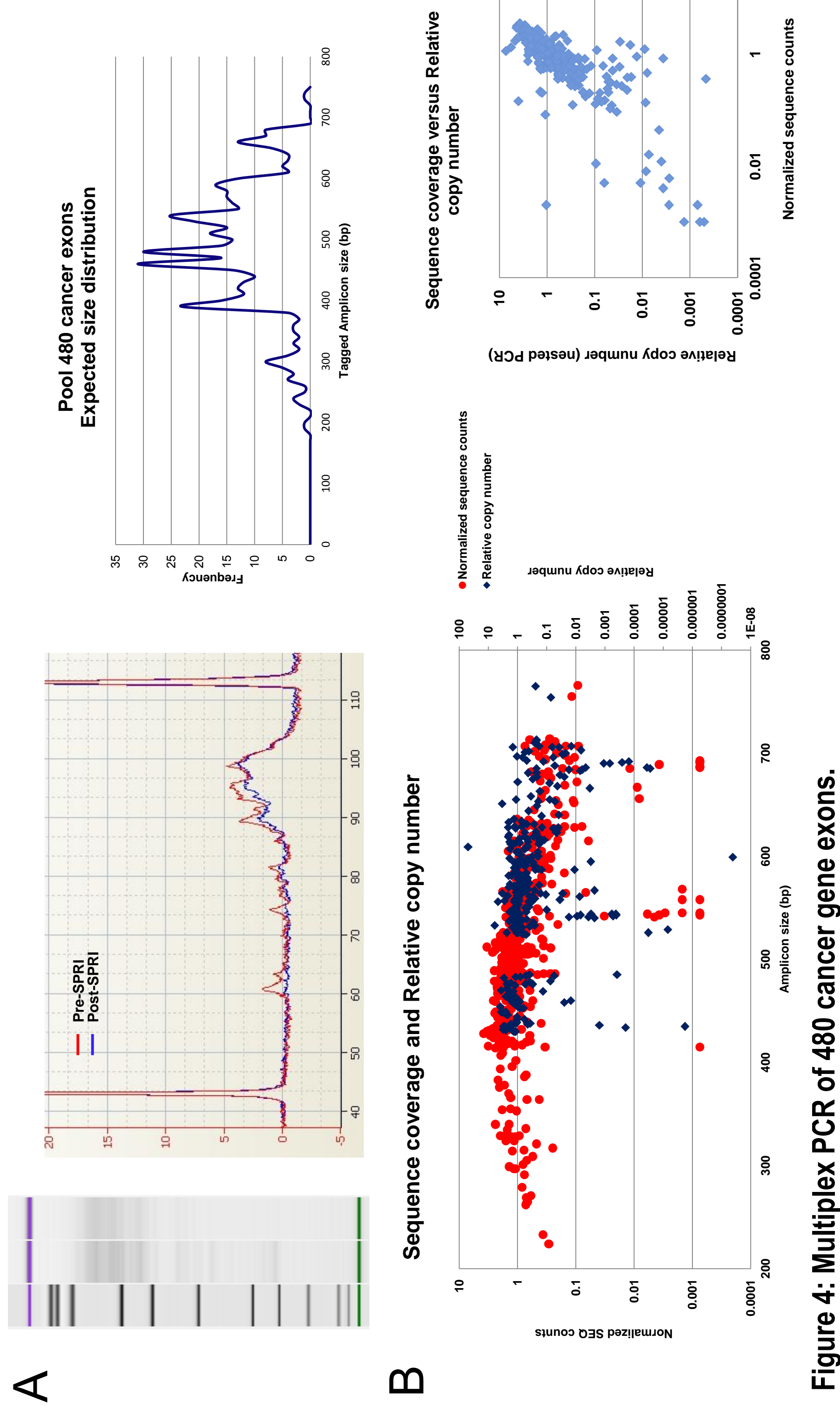


Figure 4: Multiplex PCR of 480 cancer gene exons.

Conclusion

- The Access Array System provides a simple, easy-to-use method for PCR-based target enrichment from multiple samples in parallel using only 50ng human genomic DNA per sample for up to 480 PCR reactions
- We have developed an amplicon tagging protocol that enables direct labeling of PCR products with sample-specific barcodes and sequencer-specific tags
- Data from sequencing experiments demonstrates that amplicon representation within and between samples is highly uniform
- We have demonstrated the potential for multiplexing primer pairs within PCR reactions on the Access Array system. Representation of each PCR product in the multiplex reaction is similar to that within individual PCR reactions.

Exhibit 17

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property
Organization
International Bureau



(43) International Publication Date
17 March 2005 (17.03.2005)

PCT

(10) International Publication Number
WO 2005/023091 A2

(51) International Patent Classification⁷: **A61B**

(21) International Application Number:
PCT/US2004/028857

(22) International Filing Date:
7 September 2004 (07.09.2004)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/500,526 5 September 2003 (05.09.2003) US

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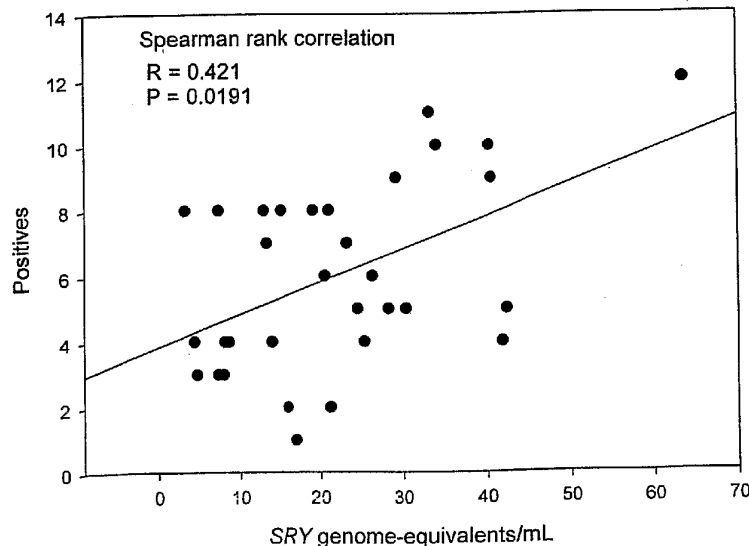
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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AT, AU, AZ, BA, BB, BG, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, HR, HU, ID, IL, IN, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MA, MD, MG, MK, MN, MW, MX, MZ, NA, NI, NO, NZ, OM, PG, PH, PL, PT, RO, RU, SC, SD, SE, SG, SK, SL, SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, YU, ZA, ZM, ZW.

(84) Designated States (unless otherwise indicated, for every kind of regional protection available): ARIPO (BW, GH,

[Continued on next page]

(54) Title: METHOD FOR NON-INVASIVE PRENATAL DIAGNOSIS



(57) Abstract: The present invention is directed to methods of detecting nucleic acids in a biological sample. The method is based on a novel combination of a base extension reaction, which provides excellent analytical specificity, and a mass spectrometric analysis, which provides excellent specificity. The method can be used, for example, for diagnostic, prognostic and treatment purposes. The method can be used, for example, for diagnostic, prognostic and treatment purposes. The method allows accurate detection of nucleic acids that are present in very small amounts in a biological sample. For example, the method of the present invention is preferably used to detect fetal nucleic acid in maternal blood sample; circulating tumor-specific nucleic acids in a blood, urine or stool sample; and donor-specific acids in transplant recipients. In another embodiment, one can detect viral, bacterial, fungal, or other foreign nucleic acids in biological sample.

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GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM, ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI, FR, GB, GR, HU, IE, IT, LU, MC, NL, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG, CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

Published:

— *without international search report and to be republished upon receipt of that report*

For two-letter codes and other abbreviations, refer to the "Guidance Notes on Codes and Abbreviations" appearing at the beginning of each regular issue of the PCT Gazette.

METHOD FOR NON-INVASIVE PRENATAL DIAGNOSIS

CROSS-REFERENCE TO RELATED APPLICATIONS

The present application claims benefit under 35 U.S.C. §119(e) of U.S. provisional patent application Serial No. 60/500,526, filed on September 5, 2003, the content of which is herein incorporated by reference in its entirety.

BACKGROUND

[001] The analysis of circulating nucleic acids has revealed applications in the noninvasive diagnosis, monitoring, and prognostication of many clinical conditions.

[002] For example, in non-invasive method fo prenatal monitoring, fetal DNA has been found to circulate in maternal plasma (Lo, Y.M.D. *et al. Lancet* **350**, 485-487 (1997)), and development of such non-invasive prenatal diagnosis has therefore been suggested based on the analysis of a maternal blood sample. Although the non-invasive nature of such approaches represents a major advantage over conventional methods. However, the technical challenge posed by the analysis of fetal DNA. Thus, in maternal plasma lies in the need to be able to discriminate the fetal DNA from the co-existing background maternal DNA, and the diagnostic reliability of circulating DNA analysis depends on the fractional concentration of the targeted sequence, the analytical sensitivity, and the specificity of the method.

[003] Fetal DNA represents a minor fraction of the total DNA in maternal plasma, contributing approximately 3% to 6% of the total maternal plasma DNA in early and late pregnancy, respectively (Lo, Y.M.D. *et al. Am J Hum Genet* **62**, 768-775 (1998)).

[004] Most diagnostic applications reported to date have focused on detecting of paternally-inherited fetal traits or mutations, as these are more readily distinguishable from

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the background maternal DNA. Reported applications include the prenatal diagnosis of sex-linked diseases (Costa, J.M., Benachi, A. & Gautier, E. *N Engl J Med* **346**, 1502 (2002)), fetal RhD status (Lo, Y.M.D. *et al. N Engl J Med* **339**, 1734-1738 (1998)) and certain paternally-transmitted autosomal dominant conditions, including achondroplasia and myotonic dystrophy (Chiu, R.W.K. & Lo, Y.M.D. *Expert Rev Mol Diagn* **2**, 32-40 (2002)).

[005] Fetal SRY and RHD DNA detection from maternal plasma has reached close to 100% accuracy, as confirmed by many large scale evaluations (Sekizawa, A., Kondo, T., Iwasaki, M., Watanabe, A., Jimbo, M., Saito, H. & Okai, T. (2001) *Clin. Chem.* **47**, 1856-1858; Finning, K. M., Martin, P. G., Soothill, P. W. & Avent, N. D. (2002) *Transfusion* **42**, 1079-1085; Costa, J. M., Benachi, A., Gautier, E., Jouannic, J. M., Ernault, P. & Dumez, Y. (2001) *Prenatal Diagn.* **21**, 1070-1074; Rijnders, R. J., Christiaens, G. C., Bossers, B., van der Smagt, J. J., van der Schoot, C. E. & de Haas, M. (2004) *Obstet. Gynecol.* **103**, 157-164). However, its general applicability is limited. The high level of diagnostic accuracy in these conditions is attained by the analytical sensitivity contributed by the use of real-time quantitative PCR (Lo Y *et al. Am. J. Hum. Genet.* **62**:768-775, 1998; Heid *et al., Genome Res.* **6**:986-994, 1996), and the analytical specificity conferred choosing fetal DNA targets that are absolutely fetal-specific. The RHD sequence does not exist in the genome of a rhesus D negative mother, and SRY, which is used to detect the presence of a Y chromosome, does not exist in a genome of a normal woman. Consequently, the maternal plasma SRY and RHD analyses are relatively free from interference by the background maternal DNA. This differs from a number of other conditions.

[006] Many fetal genetic diseases are caused by mutations that result in more subtle genetic differences between the maternal and fetal DNA sequences in maternal plasma. While such fetal diseases may theoretically be diagnosed non-invasively by means of the detection

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or exclusion of the paternally inherited mutant allele in maternal plasma, the development of robust assays for the discrimination of less dramatic differences between fetal and maternal DNA in maternal plasma has been technically challenging (Nasis, O., Thompson, S., Hong, T., Sherwood, M., Radcliffe, S., Jackson, L. & Otevrel, T. (2004) *Clin. Chem.* **50**, 694-701). Therefore, despite many potential applications reported for fetal mutation detection in maternal plasma, such as achondroplasia, Huntington's disease, cystic fibrosis, and hemoglobin E (Nasis, O., Thompson, S., Hong, T., Sherwood, M., Radcliffe, S., Jackson, L. & Otevrel, T. (2004) *Clin. Chem.* **50**, 694-701; Saito, H., Sekizawa, A., Morimoto, T., Suzuki, M. & Yanaihara, T. (2000) *Lancet* **356**, 1170; Gonzalez-Gonzalez, M. C., Trujillo, M. J., Rodriguez de Alba, M. & Ramos, C. (2003) *Neurology* **60**, 1214-1215; Gonzalez-Gonzalez, M. C., Garcia-Hoyos, M., Trujillo, M. J., Rodriguez de Alba, M., Lorda-Sanchez, I., Diaz-Recasens, J., Gallardo, E., Ayuso, C. & Ramos, C. (2002) *Prenatal Diagn.* **22**, 946-948; Fucharoen, G., Tungwiwat, W., Ratanasiri, T., Sanchaisuriya, K. & Fucharoen, S. (2003) *Prenatal Diagn.* **23**, 393-396), most published data only involve case reports of isolated patients. Large-scale evaluation of analytical protocols for circulating fetal DNA discrimination has been limited. Reliable discrimination between the fetal and maternal DNA sequences would depend heavily on the analytical specificity of the assay system. The degree of analytical specificity required for accurate analysis is inversely related to the degree of genetic difference between the alleles of interest and the background DNA (Lo, Y. M. D. (1994) *J. Pathol.* **174**, 1-6). Thus a need exists for methods that can reliably analyze such subtle genetic differences.

[007] The prenatal assessment of autosomal recessive diseases based on fetal DNA analysis in maternal plasma presents another challenge. The manifestation of an autosomal recessive disease results from the inheritance of a mutant allele from each parent. Thus, an

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autosomal recessive condition could either be confirmed prenatally through the demonstration of the inheritance of two mutant alleles, or could be excluded by the demonstration of the inheritance of at least one non-mutant allele. The current strategies look at exclusion. For example, one such strategy is based on the haplotype assessment of polymorphisms associated with the paternally-inherited non-mutant allele (Chiu, R.W.K. *et al. Clin Chem* **48**, 778-780 (2002)).

[008] β -thalassemia is an autosomal recessive condition resulting from the reduced or absent synthesis of the β -globin chains of hemoglobin. It is highly prevalent in the Mediterranean, the Middle East, the Indian subcontinent and Southeast Asia (Weatherall, D.J. & Clegg, J.B. *Bull World Health Organ* **79**, 704-712 (2001)). More than 200 β -thalassemia mutations have been described, many of which are point mutations (Weatherall, D. J. (1997) *BMJ* **314**, 1675-1678). β -thalassemia major is an otherwise lethal condition where survival is dependent on life-long blood transfusions and iron chelation therapy. Curative therapies are not readily available and therefore, much focus has been devoted to disease prevention through prenatal diagnosis.

[009] The alpha and beta loci determine the structure of the 2 types of polypeptide chains in adult hemoglobin, Hb A. Mutant beta globin that sickles causes sickle cell anemia (<http://www.ncbi.nlm.nih.gov/entrez/dispomim>). Absence of the beta chain causes beta-zero-thalassemia. Reduced amounts of detectable beta globin causes beta-plus-thalassemia, which is one of the most common single gene disorders in the world.

[010] For clinical purposes, beta-thalassemia is divided into thalassemia major (transfusion dependent), thalassemia intermedia (of intermediate severity), and thalassemia minor (asymptomatic). Patients with thalassemia major present in the first year of life have

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severe anemia; they are unable to maintain a hemoglobin level above 5 gm/dl. Clinical details of this disorder have been detailed extensively in numerous monographs and are summarized by Weatherall, et al. (The hemoglobinopathies. In: Scriver, C.; Beaudet, A. L.; Sly, W. S.; Valle, D. (eds.) : The Metabolic and Molecular Bases of Inherited Disease. (7th ed.) New York: McGraw-Hill 1995. Pp. 3417-3484). The prognosis for individuals with beta-thalassemia is very poor. For example, in 2000 it was reported that about 50% of U.K. patients with beta-thalassemia major die before the age of 35 years, mainly because conventional iron-chelation therapy is too burdensome for full adherence (Model et al. Survival in beta-thalassaemia major in the UK: data from the UK Thalassaemia Register. *Lancet* 355: 2051-2052, 2000).

[011] The molecular pathology of disorders resulting from mutations in the nonalpha-globin gene region is the best known, this elucidation having started with sickle cell anemia in the late 1940s. Steinberg and Adams reviewed the molecular defects identified in thalassemias: (1) gene deletion, e.g., of the terminal portion of the beta gene (2) chain termination (nonsense) mutations; (3) point mutation in an intervening sequence; (4) point mutation at an intervening sequence splice junction; (5) frameshift deletion; (6) fusion genes, e.g., the hemoglobins Lepore; and (7) single amino acid mutation leading to very unstable globin, e.g., Hb Vicksburg (beta 75 leu-to-0) Steinberg, M. H.; Adams, J. G., III : Thalassemia: recent insights into molecular mechanisms. *Am. J. Hemat.* 12: 81-92, 1982.

[012] Because of the frequency of the mutations in the populations and the devastating clinical symptoms including the markedly reduced life span, prenatal diagnosis is important. For example, it can provide a means for disease prevention. However, the conventional methods of prenatal diagnosis such as, amniocentesis, chorionic villus sampling and cordocentesis, are all associated with a small but finite risk of fetal loss. Therefore, it would

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be important to develop a non-invasive method for prenatal diagnosis of thalassemias. Attempts have been made in the past to develop other means of non-invasive prenatal diagnosis of β -thalassemia, including the analysis of fetal cells in maternal blood (Cheung, M.C., Goldberg, J.D. & Kan, Y.W. *Nat Genet* 14, 264-268 (1996)). However, these methods are labor-intensive and time-consuming. Consequently, the need exists to develop tools that accurately permit highly specific and sensitive detection of nucleic acids in biological samples, particularly parentally inherited alleles.

SUMMARY

[013] Accordingly, the present invention is directed to methods of detecting nucleic acids in a biological sample.

[014] We show the feasibility of the use of mass spectrometric analysis for the discrimination of fetal point mutations in maternal plasma and developed an approach for the reliable exclusion of mutations in maternal plasma. We further show the feasibility of the approach for the minimally invasive prenatal diagnosis in a situation where a mother and father share an identical disease causing mutation, a concurrence previously perceived as a challenge for maternal plasma-based prenatal diagnosis for autosomal recessive diseases.

[015] In one embodiment, the invention is directed to a method for the detection of paternally-inherited fetal-specific β -thalassemia mutations in maternal plasma based on methods for looking at nucleic acid segments using methods such as the primer-extension of polymerase chain reaction (PCR) products, at about single molecule dilution. This is preferably followed by mass spectrometric detection. The technique allows the non-invasive prenatal exclusion of β -thalassemia with high throughput capacity and is applicable to any disease caused by mutations in a single gene including, but not limited recessive single gene diseases such as thalassemias, such as beta thalassemias, cystic fibrosis, and congenital

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adrenal hyperplasia. The invention is also useful in detection of tumor-derived DNA mutations isolated from cells in the plasma of a cancer patient, and detecting donor-derived DNA in the plasma of a transplant recipient.

[016] The invention is based upon a discovery that a highly sensitive and specific mutation-specific analysis of the paternally-inherited mutation in maternal plasma can be used to exclude the fetal inheritance of the paternal mutation based on its negative detection. For example, using a real-time quantitative allele-specific polymerase chain reaction (PCR) approach to exclude the inheritance of the β -thalassemia mutation codons (CD) 41/42 (-CTTT), involving the deletion of four nucleotides (CTTT) between codons 41 and 42 of the β -globin gene, *HBB* (Chiu, R.W.K. *et al. Lancet* **360**, 998-1000 (2002)), shows that the negative exclusion proposed herein can readily be used.

[017] To achieve single nucleotide discrimination at low fractional concentrations, an analytical system that combines the use of an approach with better allele-specificity and high detection sensitivity is required. One example is the use of primer extension analysis in a system such as the MassARRAY system (SEQUENOM), that allows a high throughput approach for the detection and exclusion of paternally-inherited fetal mutations in maternal plasma with the capability of single base discrimination. The MassARRAY system is based on matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis of primer-extension products (Tang, K. *et al. Proc Natl Acad Sci USA* **96**, 10016-10020 (1999)).

[018] In one embodiment, the invention is directed to a method of detecting a genetic disorder in a fetus from a blood, serum or plasma sample of a pregnant woman, the method comprising: a) analyzing both isolated maternal and paternal DNA for a disease-causing mutation for the single gene disorder; b) if both maternal and paternal nucleic acid, e.g., DNA

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carry a disease causing mutation for the same disease then isolating the nucleic acid, e.g., DNA from plasma, blood, or serum of the pregnant mother; c) determining a fetal genotype from the isolated maternal plasma DNA using primers corresponding to the paternally identified mutation and performing a mutation-specific primer-extension assay in at least two, preferably several replicates, for example 3, 5, or about 10, 12, 15, 20, 25-100 replicates and even up to about 1000 replicates. Most preferably about 15-25 replicates are used, wherein a detection of the paternal mutation in any of the replicate sample is indicative of the presence of the single-gene disorder in the fetus.

[019] In one preferred embodiment, the single gene disease is an autosomal recessive disease. In the most preferred embodiment, the autosomal recessive disease is selected from beta thalassemias, cystic fibrosis and congenital adrenal hyperplasia. In the most preferred embodiment, the disease is beta thalassemia caused by mutations selected from the group consisting of CD 41/42 -CTTT; IVS2 654 (C->T); nucleotide -28 (A->G); and CD 17 (A->T).

[020] In the preferred embodiment the number of replicates is at least two, preferably at least about 3, 5, 10-25, or 25-100, up to at least about 1000 replicates. Most preferably the number of replicates is about 10-25.

[021] In one embodiment, the primer-extension analysis is performed using the MassARRAY system (SEQUENOM).

[022] Alternatively, the invention provides a method of detecting a genetic disease in a fetus using maternally isolated DNA from plasma, serum, or blood, the method comprising:

a) selecting one or more single nucleotide polymorphisms (SNP) which are not disease-causing polymorphisms and which are associated either with a paternal disease-causing allele or with a paternal healthy allele and which SNP differs between the maternal and the paternal

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genotype; b) determining the fetal genotype from a sample DNA isolated from the plasma, serum or whole blood of the pregnant mother, wherein the determination is performed using primers corresponding to both the selected SNP and the disease-causing mutation and performing an SNP and disease mutation-specific primer-extension assay in several replicates using said primers; c) wherein detection of the SNP associated with the paternal allele in any of the replicate samples is indicative of the presence of the paternal allele inherited by the fetus and the detection of the paternal disease-causing mutation in any of the replicate samples indicates detection of the genetic disease inherited by the fetus, wherein detection of the SNP associated with the healthy paternal allele in any of the replicate samples is indicative of the presence of the healthy allele inherited by the fetus and excludes the inheritance of the genetic disease by the fetus.

BRIEF DESCRIPTION OF DRAWINGS

[023] **Figure 1** demonstrates the relationship between the number of positives detected in the 15-replicate PCR experiments for fetal gender determination (y-axis) and the fetal DNA concentration measured by real-time quantitative PCR targeting the Y-chromosome gene, *SRY* (x-axis). The theoretical basis of using the 15-replicate format is based on the Poisson distribution of fetal DNA molecules at single molecule concentration. The equation for the Poisson distribution is:

$$[024] \quad P(n) = \frac{m^n e^{-m}}{n!}, \text{ where, } n = \text{number of fetal DNA}$$

molecules per PCR, $P(n)$ = probability of n fetal DNA molecules in a particular PCR; m = mean number of fetal DNA molecules in a particular plasma DNA sample.

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[025] Using our standard plasma DNA extraction protocol (see text), each PCR using 1 μ L of maternal plasma DNA contains 0.4 genome-equivalent of the paternally-inherited fetal DNA. Thus, the probability that a particular PCR will be negative due to having no fetal DNA molecule is:

$$[026] \quad P(0) = \frac{0.4^0 e^{-0.4}}{0!} = 0.670$$

[027] To reduce the false-negative rate, the probability that all replicates are negative for a 15-replicate PCR experiment is: $0.670^{15} = 0.0025$.

[028] **Figures 2A and 2B** show the Mass Spectrometric analyses of the SRY DNA and the paternally inherited thalassemia IVS2 654 mutation. Mass spectra for the other three thalassemia mutations are similar. For all mass spectra, Mass (x-axis) represents the molecular weight of the marked peaks. The molecular weights of all relevant peaks are calculated before the analysis and the Mass values measured by mass spectrometry are generally only 0-5 Dalton off. Intensity (y-axis) is of an arbitrary unit. P and PP represent unextended primer and pausing product (i.e., premature termination of the base extension reaction), respectively. For SRY DNA analysis, the SRY peak is present (thus a positive result, marked as POS at the left side of the figure) in some of the 15 replicates (see Fig. 1). None of the 15 replicates has a SRY peak (thus a negative result, marked as NEG in the figure), if a women was pregnant with a female fetus. For thalassemia IVS2 654 mutation analysis, the pT peak is from the paternally inherited thalassemia IVS2 654 mutation and is present in some of the 15 replicates for fetuses carrying a paternally inherited thalassemia IVS2 654 mutation (see Table 1). The nP peak is from all other β -globin alleles except the paternally inherited thalassemia IVS2 654 allele.

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[029] **Figure 3** shows a schematic illustration of the single allele base extension reaction (SABER) and standard MassARRAY assays. Maternal plasma detection of the paternally inherited fetal-specific β -thalassemia mutation, IVS2 654 C \rightarrow T, is presented as an illustrative example. Maternal plasma is first amplified by PCR. The PCR products are subjected to base extension by the standard and SABER protocols. The standard protocol involves the base extension of both the mutant fetal allele (T allele) and the background allele (C allele), whereas the SABER method only extends the fetal-specific mutant allele. The base extension reactions are terminated by dideoxynucleotides, indicated in boxes. The extension products of the standard protocol include a predominance of the nonmutant allele (open arrows) with a small fraction of the fetal-specific mutant allele (filled arrows). The low abundance of the fetal allele (filled peak) is overshadowed by the nonmutant allele (open peak) on the mass spectrum. Because SABER only involves the extension of the mutant allele, the latter's presence (filled peak) can be robustly identified from the mass spectrum. The striped peaks represent the unextended primer.

[030] **Figures 4A-4D** show the MS analyses of the paternally inherited β -thalassemia IVS2 654 mutation in maternal plasma. For all mass spectra, mass (x axis) represents the molecular weight of the marked peaks. The expected molecular weights of all relevant peaks were calculated before the analysis. Intensity (y axis) is in arbitrary units. P and PP, unextended primer and pausing product (i.e., premature termination of the base extension reaction or incorporation of an undigested dGTP from shrimp alkaline phosphatase treatment for the wild-type DNA template), respectively. Figs 4A and 4B illustrate the mass spectra obtained by the standard MassARRAY protocol for a fetus negative and positive for the mutation, respectively. T, expected mass of the mutant allele; C, position of the alleles without the IVS2 654 mutation. Figs 4C and 4D illustrate the mass spectra obtained by the

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SABER MassARRAY protocol for a fetus negative and positive for the mutation, respectively. IVS2 654, expected mass of the mutant allele.

[031] **Figure 5** shows Table 1, showing prenatal exclusion of β -thalassemia major by maternal plasma analysis. All of the parents are carriers for β -thalassemia and have one *HBB* mutation. The ^amutations of the father and mother are marked by "F" and "M", respectively. The maternal mutation is not indicated for cases where the maternal mutation is not one of the four *HBB* mutations studied. The ^bfetal genotype is indicated by the inheritance of the paternal mutation "F", the maternal mutation "M", or the normal allele "*". Results of the MassARRAY maternal plasma analysis is indicated by the ^cnumber of replicates among the 15 repeats where the paternally-inherited fetal allele was positively detected. The fetus is deemed to have inherited the paternal mutation if any of the 15 repeats showed a positive result.

[032] **Figure 6** shows Table 2 including PCR and extension primer sequences. *CCT mix is ddCTP/ddGTP/ddTTP/dATP in which dd indicates the 2',3'-dideoxynucleoside. Similarly AC mis is ddATP/ddCTP/dGTP/dTTP.

[033] **Figure 7** shows Table 3 including data from detection of paternally inherited *HBB* mutations in maternal plasma. All the patients are carriers of β -thalassemia and have one *HBB* mutation. The maternal mutation is not indicated for cases where the maternal mutation is not one of the four *HBB* mutations studies. F and M, mutations of the father and mother, respectively; -, no mutation; neg, negative; pos, positive; N.A. not applicable. †The fetal genotype determined by conventional methods is indicated by the inheritance of the paternal mutation F, the maternal mutation M, or the normal allele, *.

[034] **Figure 8** shows Table 4 including data from haplotype analysis of paternally inherited alleles in maternal plasma. Neg, negative; Pos, positive; N.A., not applicable. †G

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and C denote the rs2187610 allele linked to the mutant or wild-type paternal *HBB* alleles, respectively. ‡ The fetal genotype determined by conventional methods is indicated by the inheritance of the paternal mutation F, the maternal mutation M, or the normal allele, *.

DETAILED DESCRIPTION

[035] The present invention is directed to methods of detecting nucleic acids in a biological sample. The method is based on a novel combination of a base extension reaction, which provides excellent analytical specificity, and a mass spectrometric analysis, which provides excellent specificity. The method can be used, for example, for diagnostic, prognostic and treatment purposes. The method allows accurate detection of nucleic acids that are present in very small amounts in a biological sample. For example, the method of the present invention is preferably used to detect fetal nucleic acid in a maternal blood sample; circulating tumor-specific nucleic acids in a blood, urine or stool sample; and donor-specific nucleic acids in transplant recipients. In another embodiment, one can detect viral, bacterial, fungal, or other foreign nucleic acids in a biological sample.

[036] The methods provided are minimally invasive, requiring generally, a small amount of a biological sample, for example, a blood, plasma, serum, urine, buccal or nasal swap, saliva, skin scratch, hair or stool sample from an individual.

[037] In the case of determining a fetal genotype or quantitating the fetal nucleic acids or alleles using the methods of the present invention, the sample can be any maternal tissue sample available without posing a risk to the fetus. Such biological materials include maternal blood, plasma, serum, saliva, cerebrospinal fluid, urine or stool samples.

[038] In the present study, we evaluated, and show the feasibility of, the use of mass spectrometry (MS) for the discrimination of fetal point mutations in maternal plasma and

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developed an approach for the reliable exclusion of mutations in maternal plasma. We further evaluated, and show the feasibility of, the approach for the noninvasive prenatal diagnosis of a mother and father sharing an identical disease causing mutation, an occurrence previously perceived as a challenge for maternal plasma-based prenatal diagnosis for autosomal recessive diseases.

[039] The methods of the present invention are automatable. For example, use of mass spectrometry, such as MassARRAY system (Sequenom Inc, CA), in combination with the present invention allows analysis of fetal DNA with the capacity of over 2000 samples per day in triplicate samples thus making the method a practical system for routine use.

[040] In one preferred embodiment, the invention provides an accurate method for determining differences between fetal and maternal nucleic acids in a maternal blood sample allowing for a minimally invasive and reliable method for prenatal diagnosis. The method is based on a combination of a base extension reaction and a mass spectrometric analysis.

Thus, prenatal diagnosis can be performed without the potential complications to the fetus and the mother that are associated with traditional methods for prenatal diagnosis including amniotic fluid and/or chorionic villus sampling.

[041] Due to the specificity of the base extension reaction, allelic differences can be accurately amplified for analysis including changes varying from single nucleotide variations to small and large deletions, insertions, inversions and other types of nucleic acid changes that occur in even a small percentage of the pool of nucleic acids present in a sample.

[042] The base extension reaction according to the present invention can be performed using any standard base extension method. In general, a nucleic acid primer is designed to anneal to the target nucleic acid next to or close to a site that differs between the different alleles in the locus. In the standard base extension methods, all the alleles present in the

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biological sample are amplified, when the base extension is performed using a polymerase and a mixture of deoxy- and dideoxynucleosides corresponding to all relevant alleles. Thus, for example, if the allelic variation is A/C, and the primer is designed to anneal immediately before the variation site, a mixture of ddATP/ddCTP/dTTP/dGTP will allow amplification of both of the alleles in the sample, if both alleles are present. Table 2 in Figure 6 shows exemplary mixtures for the standard base extension reactions for detecting several different nucleic acid variations in the *HBB* locus.

[043] The After the base extension reaction, the extension products including nucleic acids with A and C in their 3' ends, can be separated based on their different masses. Alternatively, if the ddNTPs are labeled with different labels, such as radioactive or fluorescent labels, the alleles can be differentiated based on the label. In a preferred embodiment, the base extension products are separated using mass spectrometric analysis wherein the peaks representing different masses of the extension products, represent the different alleles.

[044] In one embodiment, the base extension is performed using single allele base extension reaction (SABER, Fig. 3). In SABER, one allele of interest per locus is amplified in one reaction by adding only one dideoxynucleotide corresponding to the allele that one wishes to detect in the sample. One or more reactions can be performed to determine the presence of a variety of alleles in the same locus. Alternatively, several loci with one selected allele of interest can be extended in one reaction.

[045] The specificity provided by primer extension reaction, particularly SABER, allows accurate detection of nucleic acids with even a single base pair difference in a sample, wherein the nucleic acid with the single base pair difference is present in very small amounts. For example, fetal nucleic acids have been generally found to represent only about 3-6% of

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the nucleic acids circulating in the maternal blood (Lo et al, Am J Hum Genet 62, 768-775, 1998). We have now shown that the methods provided by the present invention can be used to detect polymorphisms present in the fetal nucleic acids from a sample taken from the pregnant mother.

[046] Therefore, in one embodiment, the invention provides a method for detecting fetal nucleic acids in maternal blood. The method comprises obtaining a nucleic acid sample from the pregnant mother and analyzing the sample using base extension and subsequent mass spectrometric analysis to detect one or more loci of the fetal nucleic acid in the sample.

[047] In one embodiment, the invention provides a method for detecting a paternally-inherited mutations in the fetus from the maternal blood. The method comprises analyzing the paternal nucleic acid sample and determining the presence of one or more paternal nucleic acid polymorphisms. The maternal blood/plasma sample is then analyzed for the presence or absence of the paternally inherited allele using base extension, preferably SABER, wherein only ddNTP corresponding to the paternal mutation(s) is used in the base extension reaction. The base extension products are then detected using any detection methods, that can differentiate between the base extended nucleic acid products. Preferably, the detection is performed using matrix assisted laser desorption ionization/time-of-flight mass spectrometric analysis, for example, as described in Example 2. The presence and/or absence of the paternal alleles in the maternal blood/plasma sample is exemplified in Figure 4, wherein the presence of the peak representing the paternally inherited IVS2 654 (C→T) can be seen in Fig. 4D and absence of the same allele in Fig. 4C.

[048] The method can be used to reliably detect paternally inherited disease causing mutations including any dominant or recessive diseases such as achondroplasia, Huntington's disease, cystic fibrosis, hemoglobin E and the different hemoglobinopathies, such as β -

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thalassemia. Based on this disclosure, a skilled artisan will be able to design a detection method for prenatal diagnosis for any disease wherein the disease causing mutation or a genetic polymorphism(s) associated with or linked to the disease is/are known.

[049] We have illustrated the reliability of the SABER assays for single-nucleotide discrimination between circulating fetal and maternal DNA by the maternal plasma detection of fetal β -thalassemia point mutations and SNPs. The ability to robustly analyze fetal-specific SNPs in maternal plasma is a useful adjunct procedure for maternal-plasma fetal DNA analysis as a safeguard against the possibility of false-negative detection due to fetal DNA degradation, DNA extraction failures, or PCR allele dropout. With the availability of a reliable MS method for fetal single nucleotide polymorphism (SNP) detection in maternal plasma, the number of potential gender-independent internal control targets for circulating fetal DNA detection has increased substantially.

[050] Circulating fetal SNPs can also be analyzed according to the method of the present invention,. This permits fetal haplotype analysis from maternal plasma. Noninvasive fetal haplotyping can be achieved by means of analyzing polymorphisms linked to a mutated locus. Haplotype analysis between the *HBB* locus and a linked polymorphism permits the noninvasive prenatal exclusion of β -thalassemia major, despite the presence of the same *HBB* mutation in both parents. See, for example, case 12 of Example 2 below. This procedure overcomes the previously perceived deficiency in maternal plasma-based prenatal diagnosis of autosomal recessive diseases that limited its applicability to couples sharing different mutations.

[051] Therefore, the haplotype approach also can be applied to maternal plasma detection of a fetal SNP allele linked to the paternal nonmutant allele. The positive detection of such an allele results in the noninvasive positive prenatal exclusion of a disease, such as β -

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thalassemia major and other recessive diseases wherein mother and father share the same mutation but carry different SNPs. Example 2 shows the results of non-invasive fetal haplotyping in a β -thalassemia case using an informative polymorphism at locus rs2187610.

[052] Thus, the invention provides a method for determining a fetal haplotype to determine the presence of paternally inherited allele at any given locus in the fetal genome from the maternal plasma/blood. The method comprises the steps of determining one or more polymorphisms that differ between maternal and paternal genomes, i.e., SNPs that are informative. The SNPs should be linked to the locus wherein determination of any given allele is desired. The determination of informative SNPs can be performed using any genotyping methods routinely available for a skilled artisan. Any haplotyping methods can be used. In one preferred embodiment, direct molecular haplotyping method is used as explained below (see, Ding and Cantor, Proc Natl Acad Sci U S A, 100: 7449-7453, 2003). The fetal nucleic acid is analyzed from the maternal blood/plasma using the method of the present invention comprising amplification of the nucleic acids, for example using PCR, performing a base extension reaction, preferably SABER, followed by detection of the base extension products, preferably using MS based techniques.

[053] Haplotyping of the fetal nucleic acid allows accurate prenatal diagnosis of a disease wherein both parents may be carriers of the same mutation, but carry that mutation in only one allele. Therefore, for example, if the disease is recessive, determination of one healthy allele in the fetal nucleic acid shows that the fetus, if born, will not be affected with the disease.

[054] In one embodiment, the method of the invention is applied to quantification of fetal nucleic acids from the maternal biological sample. Quantitative aberrations in circulating fetal DNA concentrations have been demonstrated for fetal chromosomal

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aneuploidies, preeclampsia, preterm labor, and many other pregnancy associated complications. Therefore, the present method permits for determining the risk of aneuploidies, preeclampsia, preterm labor, and other pregnancy associated complications. The methods use the principle of analyzing the presence of fetal specific paternally inherited allele determined using nucleic acid amplification, base extension and analysis of the base extension products as described elsewhere in the specification. Quantification is consequently performed either by comparing the ratio of the maternal allele and the fetal specific allele or by including an external standard in known amounts to determine the amount or relative amount of the fetal nucleic acid in the sample. Because the method of the invention allows minimally invasive sample collection, the comparison can be performed either at one desired time during pregnancy or several times during the parts or entire pregnancy to allow a follow-up of the fetal condition throughout the pregnancy.

[055] In one embodiment, the invention provides a method for the detection of paternally-inherited fetal-specific β -thalassemia mutations in maternal plasma based on a method of analyzing the paternal nucleic acid at single molecule dilution. Preferably, the analysis is performed using a primer-extension of polymerase chain reaction (PCR) and detecting the primer extension products using mass spectrometry. Alternatively, the detection can be performed using, for example, electrophoretic methods including capillary electrophoresis, using denaturing high performance liquid chromatography (D-HPLC), using an Invader® Assay (Third Wave Technologies, Inc., Madison, Wis.), pyrosequencing techniques (Pyrosequencing, Inc., Westborough, MA) or solid-phase minisequencing (U.S. Patent No. 6,013,431, Suomalainen et al. Mol. Biotechnol. Jun;15(2):123-31, 2000).

[056] In one embodiment the invention is directed to a method of detecting a genetic disorder in a fetus from a plasma sample of a pregnant woman, the method comprising: a)

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analyzing both maternal and paternal nucleic acid, e.g., DNA for a disease-causing mutation for the single gene disorder; b) if both maternal and paternal nucleic acid, e.g., DNA or RNA, preferably DNA, carry a disease causing mutation for the same disease then isolating nucleic acid, e.g., DNA isolated from blood, plasma or serum of the pregnant mother; c) determining a fetal genotype from the isolated maternal plasma nucleic acid, e.g., DNA using primers corresponding to the paternally identified mutation and performing a mutation-specific primer-extension assay, preferably in several replicates, wherein a detection of the paternal mutation in any of the replicate sample is indicative of the presence of the single-gene disorder in the fetus.

[057] The gestational age of the fetus preferably varies from about 7 to about 23 weeks.

[058] The genetic disease according to the present invention may be any disease wherein a disease causing mutation is known or wherein a genetic polymorphisms associated with or linked to the disease are known. Preferably the disease is one caused by mutations in one gene and most preferably, the diseases is a recessive single gene disease. The mutations can vary from single nucleotide point mutations to insertions, inversions, and deletions of any number of nucleotides in the genomic DNA. Preferably, the recessive genetic disease is caused by two different mutations wherein one is inherited from the mother and the other from the father. Preferred examples of genetic diseases that can be diagnosed using the method of the present invention include but are not limited to thalassemias, such as beta thalassemias, cystic fibrosis, and congenital adrenal hyperplasia.

[059] DNA isolation from blood, plasma, or serum can be performed using any method known to one skilled in the art. One such method is disclose in Chiu, R.W.K. *et al. Clin Chem* 47:1607-1613. (2001) incorporated herein by reference in its entirety. Other suitable methods include, for example TRI REAGENT® BD (Molecular Research Center, Inc.,

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Cincinnati, OH), which is a reagent for isolation of DNA from, for example, plasma. TRI REAGENT BD and the single-step method are described, for example, in the US Patent Nos. 4,843,155 and 5,346,994.

[060] Different alleles present in the nucleic acid sample are consequently either amplified using PCR and then differentiated using various differential amplification methods described below, including different primer extension methods. Alternatively, the different alleles are amplified and differentiated simultaneously, for example using the below-described INVADER assay.

[061] In one embodiment, before the primer extension reaction the isolated DNA is amplified using PCR and primers flanking the known mutation site and/or the single nucleotide polymorphism (SNP) site. In one preferred embodiment, primers presented in Table 2 are used to detect the corresponding beta thalassemia mutations shown in the Table 2. In an alternative embodiments, no pre-amplification of the sample is necessary.

[062] In one embodiment, a primer extension reaction is used to detect or “enhance” or “amplify” or “highlight” the different alleles present in the maternal nucleic acid sample. Primer extension reaction can be performed using any protocol for primer extension known to one skilled in the art (see, e.g., Molecular Cloning: A Laboratory Manual, 3rd Ed., Sambrook and Russel, Cold Spring Harbor Laboratory Press, 2001) .

[063] For example, methods including complementary DNA (cDNA) arrays (Shalon et al., Genome Research 6(7):639-45, 1996; Bernard et al., Nucleic Acids Research 24(8):1435-42, 1996), solid-phase mini-sequencing technique (U.S. Patent No. 6,013,431, Suomalainen et al. Mol. Biotechnol. Jun;15(2):123-31, 2000), ion-pair high-performance liquid chromatography (Doris et al. J. Chromatogr. A May 8;806(1):47-60, 1998), and 5' nuclease

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assay or real-time RT-PCR (Holland et al. Proc Natl Acad Sci USA 88: 7276–7280, 1991), or primer extension methods described in the U.S. Patent No. 6,355,433, can be used.

[064] In one embodiment, the primer extension reaction and analysis is performed using PYROSEQUENCING™ (Uppsala, Sweden) which essentially is sequencing by synthesis. A sequencing primer, designed directly next to the nucleic acid differing between the disease-causing mutation and the normal allele or the different SNP alleles is first hybridized to a single stranded, PCR amplified DNA template from the mother, and incubated with the enzymes, DNA polymerase, ATP sulfurylase, luciferase and apyrase, and the substrates, adenosine 5' phosphosulfate (APS) and luciferin. One of four deoxynucleotide triphosphates (dNTP), for example, corresponding to the nucleotide present in the disease-causing allele, is then added to the reaction. DNA polymerase catalyzes the incorporation of the dNTP into the standard DNA strand. Each incorporation event is accompanied by release of pyrophosphate (PPi) in a quantity equimolar to the amount of incorporated nucleotide. Consequently, ATP sulfurylase converts PPi to ATP in the presence of adenosine 5' phosphosulfate. This ATP drives the luciferase-mediated conversion of luciferin to oxyluciferin that generates visible light in amounts that are proportional to the amount of ATP. The light produced in the luciferase-catalyzed reaction is detected by a charge coupled device (CCD) camera and seen as a peak in a PYROGRAM™. Each light signal is proportional to the number of nucleotides incorporated and allows a clear determination of the presence or absence of, for example, the disease causing allele. Thereafter, apyrase, a nucleotide degrading enzyme, continuously degrades unincorporated dNTPs and excess ATP. When degradation is complete, another dNTP is added which corresponds to the dNTP present in for example the selected SNP. Addition of dNTPs is performed one at a time. Deoxyadenosine alfa-thio triphosphate (dATP α S) is used as a substitute for the natural deoxyadenosine triphosphate (dATP) since it

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is efficiently used by the DNA polymerase, but not recognized by the luciferase. For detailed information about reaction conditions for the PYROSEQUENCING, see, e.g. U.S. Patent No. 6,210,891, which is herein incorporated by reference in its entirety.

[065] Another example of the methods useful for detecting the different alleles in the sample isolated from the maternal plasma, serum or blood, is real time PCR. All real-time PCR systems rely upon the detection and quantification of a fluorescent reporter, the signal of which increases in direct proportion to the amount of PCR product in a reaction. Examples of real-time PCR method useful according to the present invention include, TaqMan® and molecular beacons, both of which are hybridization probes relying on fluorescence resonance energy transfer (FRET) for quantitation. TaqMan Probes are oligonucleotides that contain a fluorescent dye, typically on the 5' base, and a quenching dye, typically located on the 3' base. When irradiated, the excited fluorescent dye transfers energy to the nearby quenching dye molecule rather than fluorescing, resulting in a nonfluorescent substrate. TaqMan probes are designed to hybridize to an internal region of a PCR product (ABI 7700 (TaqMan™), Applied BioSystems, Foster City, CA). Accordingly, two different primers, one hybridizing to the disease-causing allele and the other to the selected SNP allele nucleic acid template, are designed. The primers are consequently allowed to hybridize to the corresponding nucleic acids in the real time PCR reaction. During PCR, when the polymerase replicates a template on which a TaqMan probe is bound, the 5' exonuclease activity of the polymerase cleaves the probe. Consequently, this separates the fluorescent and quenching dyes and FRET no longer occurs. Fluorescence increases in each cycle, proportional to the rate of probe cleavage.

[066] Molecular beacons also contain fluorescent and quenching dyes, but FRET only occurs when the quenching dye is directly adjacent to the fluorescent dye. Molecular beacons are designed to adopt a hairpin structure while free in solution, bringing the fluorescent dye

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and quencher in close proximity. Therefore, for example, two different molecular beacons are designed, one recognizing the disease-causing allele and the other the selected SNP nucleic acid. When the molecular beacons hybridize to the nucleic acids, the fluorescent dye and quencher are separated, FRET does not occur, and the fluorescent dye emits light upon irradiation. Unlike TaqMan probes, molecular beacons are designed to remain intact during the amplification reaction, and must rebind to target in every cycle for signal measurement. TaqMan probes and molecular beacons allow multiple DNA species to be measured in the same sample (multiplex PCR), since fluorescent dyes with different emission spectra may be attached to the different probes, e.g. different dyes are used in making the probes for different disease-causing and SNP alleles. Multiplex PCR also allows internal controls to be co-amplified and permits allele discrimination in single-tube assays. (Ambion Inc, Austin, TX, TechNotes 8(1) - February 2001, Real-time PCR goes prime time).

[067] Yet another method useful according to the present invention for emphasizing or enhancing the difference between the disease-causing and normal allele and the different selected SNP alleles is solid-phase mini-sequencing (Hultman, et al., 1988, Nucl. Acid. Res., 17, 4937-4946; Syvanen et al., 1990, Genomics, 8, 684-692). In the original reports, the incorporation of a radiolabeled nucleotide was measured and used for analysis of the three-allelic polymorphism of the human apolipoprotein E gene. The method of detection of the variable nucleotide(s) is based on primer extension and incorporation of detectable nucleoside triphosphates in the detection step. By selecting the detection step primers from the region immediately adjacent to the variable nucleotide, this variation can be detected after incorporation of as few as one nucleoside triphosphate. Labelled nucleoside triphosphates matching the variable nucleotide are added and the incorporation of a label into the detection step primer is measured. The detection step primer is annealed to the copies of the target

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nucleic acid and a solution containing one or more nucleoside triphosphates including at least one labeled or modified nucleoside triphosphate, is added together with a polymerizing agent in conditions favoring primer extension. Either labeled deoxyribonucleoside triphosphates (dNTPs) or chain terminating dideoxyribonucleoside triphosphates (ddNTPs) can be used. The solid-phase mini-sequencing method is described in detail, for example, in the U.S. Patent No. 6,013,431 and in Wartiovaara and Syvanen, Quantitative analysis of human DNA sequences by PCR and solid-phase minisequencing. Mol Biotechnol 2000 Jun; 15(2):123-131.

[068] Another method to detect the different alleles in the PCR products from the maternal sample is by using fluorescence tagged dNTP/ddNTPs. In addition to use of the fluorescent label in the solid phase mini-sequencing method, a standard nucleic acid sequencing gel can be used to detect the fluorescent label incorporated into the PCR amplification product. A sequencing primer is designed to anneal next to the base differentiating the disease-causing and normal allele or the selected SNP alleles. A primer extension reaction is performed using chain terminating dideoxyribonucleoside triphosphates (ddNTPs) labeled with a fluorescent dye, one label attached to the ddNTP to be added to the standard nucleic acid and another to the ddNTP to be added to the target nucleic acid.

[069] Alternatively, an INVADER[®] assay can be used (Third Wave Technologies, Inc (Madison, WI)). This assay is generally based upon a structure-specific nuclease activity of a variety of enzymes, which are used to cleave a target-dependent cleavage structure, thereby indicating the presence of specific nucleic acid sequences or specific variations thereof in a sample (see, e.g. U.S. Patent No. 6,458,535). For example, an INVADER[®] operating system (OS), provides a method for detecting and quantifying DNA and RNA. The INVADER[®] OS is based on a "perfect match" enzyme-substrate reaction. The INVADER[®] OS uses

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proprietary CLEAVASE[®] enzymes (Third Wave Technologies, Inc (Madison, WI)), which recognize and cut only the specific structure formed during the INVADER[®] process which structure differs between the different alleles selected for detection, i.e. the disease-causing allele and the normal allele as well as between the different selected SNPs. Unlike the PCR-based methods, the INVADER[®] OS relies on linear amplification of the signal generated by the INVADER[®] process, rather than on exponential amplification of the target.

[070] In the INVADER[®] process, two short DNA probes hybridize to the target to form a structure recognized by the CLEAVASE[®] enzyme. The enzyme then cuts one of the probes to release a short DNA "flap." Each released flap binds to a fluorescently-labeled probe and forms another cleavage structure. When the CLEAVASE[®] enzyme cuts the labeled probe, the probe emits a detectable fluorescence signal.

[071] Disease-causing alleles and the SNPs may also be differentiated using allele-specific hybridization followed by a MALDI-TOF-MS detection of the different hybridization products.

[072] In the preferred embodiment, the detection of the enhanced or amplified nucleic acids representing the different alleles is performed using matrix-assisted laser desorption ionization/time-of-flight (MALDI-TOF) mass spectrometric (MS) analysis described in the Examples below. This method differentiates the alleles based on their different mass and can be applied to analyze the products from the various above-described primer-extension methods or the INVADER[®] process.

[073] It has been shown that during early pregnancy, the median total DNA concentration in maternal plasma is approximately 1000 genome-equivalents per milliliter (Lo, Y.M.D., et al. *Am J Hum Genet* 62,768-775, 1998). Fetal DNA comprises some 5% of the total DNA in maternal plasma and theoretically, half of which is contributed by the

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paternally-inherited fraction. Using maternal plasma DNA extraction and PCR protocols as previously described (Lo, Y.M.D., et al. *Am J Hum Genet* 62,768-775, 1998) (for example, DNA extraction from about 800 μ L of maternal plasma eluted in about 50 μ L water, and using about 5 μ L of DNA per PCR), each reaction will theoretically contain about 2 copies of the paternally-derived fetal DNA (i.e., in the example above, $1000 \times 0.8 \times 2.5\% \times 5 / 50$) and about 76 copies of maternal DNA (i.e., in the example above, $1000 \times 0.8 \times 95\% \times 5 / 50$).

[074] The low fractional concentration poses significant demands on the sensitivity and specificity required for the analytical system. In addition, due to the low absolute concentration, fetal DNA fragments in maternal plasma are distributed stochastically (Ding, C & Cantor, C.R., *Proc Natl Acad Sci U S A* 100, 7449-7453, 2003). If analyses were performed in multiple replicates, each replicate would either contain no or only some fetal DNA fragments. The proportion of replicates that does contain fetal DNA fragments is therefore governed by the Poisson distribution. If the experiments were performed at further dilution, for example, using 1 μ L of plasma DNA, the amount of maternal DNA per replicate will reduce to one-fifth and for replicates that contain one copy of the fetal-specific paternal allele, the fractional concentration of the fetal fraction will increase from 2.5% (2/80) to 6.25%(1/16), thus, improving the robustness for fetal DNA detection and allelic discrimination.

[075] Therefore, it is important to use several replicates of the analysis. In a preferred embodiment, at least about 10, preferably 15 or more replicates up to about 20, 50, 70 or 100 replicates are used to improve statistical accuracy of the allele determination.

[076] In another embodiment, the invention provides a method wherein single nucleotide polymorphism (SNP) detection is incorporated with the simultaneous detection of

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the mutated or healthy alleles. SNPs that are associated with either the mutated or normal paternal allele can be used in this embodiment.

[077] The additional assessment of SNPs will also help to eliminate the false-negative results, as a diagnostic result is only regarded as valid if a paternal SNP allele is detected in at least one of the replicates.

[078] Although the MassARRAY system was originally designed for high-throughput SNP detection (Tang K., et al., *Proc Natl Acad Sci U S A* 96, 10016-10020, 1999), the discrimination of paternal and maternal mutant alleles that share the same mutation can be achieved by the detection of SNPs that are linked particularly to the paternal mutation which combination provides an improved method for non-invasive prenatal diagnosis of recessive diseases. The detection of SNPs associated with the paternal normal or healthy allele instead, would, for example, allow the positive exclusion of β -thalassemia (Chiu, R.W.K et al., *Clin Chem* 48, 778-780, 2002) in the fetus by using a maternal plasma sample.

[079] The present method can also provide a tool to analyze other circulating nucleic acids including, but not limited to tumor-specific nucleic acid changes, viral, bacterial, fungal, and protozoan nucleic acids, and donor-specific nucleic acids in a transplant patient. The method comprises analyzing nucleic acids in a biological sample by primer extension to differentiate between the normal, or host nucleic acids and the mutant, or non-host nucleic acids, and analyzing the primer extension products, preferably using mass spectrometry.

[080] In one embodiment, the method is used to follow development of mutant forms of, for example, tumor cells, viruses and bacteria in an individual, thus providing an easy and minimally invasive method to detect development of, for example drug-resistant tumor cells and antibiotic resistant bacteria.

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[081] All references cited herein and throughout the specification are incorporated herein by reference in their entirety.

EXAMPLES

Example 1

[082] The feasibility of assessing fetal gender from maternal plasma using the MassARRAY system is shown herein. Forty-one normal pregnancies comprising 32 male and 9 female fetuses were recruited with informed consent. Ten milliliters of maternal blood was collected. Maternal plasma was obtained and DNA was extracted as previously described (Chiu, R.W.K. *et al. Clin Chem* **47**, 1607-1613. (2001)). Fetal gender was determined in the maternal plasma by MassARRAY analysis of the primer extension products of a Y-chromosome-specific PCR and a previously developed real-time quantitative PCR assay (Lo, Y.M.D. *et al. Am J Hum Genet* **62**, 768-775 (1998)). For MassARRAY analysis, one microliter of maternal plasma DNA was used in each five microliter PCR reaction. After removing excess dNTPs with a shrimp alkaline phosphatase, base extension reaction was carried out (PCR and extension primer sequences are provided in supplementary table 1). The extension products were analyzed by MALDI-TOF mass spectrometry (SEQUENOM) (Ding, C. & Cantor, C.R. *Proc Natl Acad Sci U S A* **100**, 7449-7453 (2003)). Fifteen replicates were performed for each sample. The number of replicates was determined by probabilistic calculations based on the Poisson distribution (Fig. 1) to maximize the probability of the positive detection of the fetal DNA molecules in any of the replicates. The fetal gender was reported as male if any of the 15 replicates was positive for the Y-chromosome-specific product. The fetal gender was correctly predicted in all cases. The proportion of replicates with positive Y-chromosome product correlated with that predicted

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by the theoretical estimates of the Poisson distribution based on the fetal DNA concentration determined by real-time quantitative PCR (Spearman rank correlation $R=0.421$; $p=0.0191$) (Fig. 1).

[083] As shown below, the approach allows single nucleotide discrimination between fetal and maternal DNA. We studied pregnancies where the fetus was at risk for β -thalassemia major. The protocol was adopted for maternal plasma analysis of the paternally-inherited fetal-specific *HBB* mutation for the four most common Southeast Asian β -thalassemia mutations. These four mutations included CD 41/42 -CTTT, IVS2 654 (C→T), nt -28 (A→G) and CD 17 (A→T), and they account for about 90% of all β -thalassemia mutations in Southeast Asia (Lau, Y.L. *et al. N Engl J Med* **336**, 1298-1301 (1997)).

[084] Twenty-three couples whose pregnancies were at risk of β -thalassemia major were recruited with institutional consent from the established prenatal diagnostic centers at The Chinese University of Hong Kong, Hong Kong; Chiang Mai University, Thailand; and KK Women's and Children's Hospital, Singapore. Ten milliliters of maternal and paternal blood were collected into EDTA tubes prior to chorionic villus sampling, amniocentesis or cordocentesis. The median gestational age at the time of sampling was about 17 weeks (ranging from about 8 to about 22 weeks).

[085] Parental genotype and mutation analyses were performed according to established diagnostic practices. The 23 pregnancies were fathered by 11 carriers of CD 41/42 -CTTT mutation, 6 carriers of IVS2 654 (C→T) mutation, 1 carrier of nt -28 (A→G) mutation and 5 individuals who were carriers of the CD 17 (A→T) mutation (Fig. 5, Table 1).

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[086] As a control, fetal genotype was determined by molecular analysis of chorionic villi, amniotic fluid or fetal whole blood. The researcher who analyzed the maternal plasma samples was blinded from the fetal genotype result.

[087] Primer extension and MassARRAY analysis were performed essentially in the same manner as the fetal gender experiments with the exception that the fetal targets for detection were the paternally-inherited *HBB* mutations. Four PCR and primer-extension assays were designed and these corresponded to each of the four mutations. The PCR and extension primer sequences are provided in Table 1 (Fig. 6). The assay primers used in a particular sample was selected according to the mutation that the father carried.

[088] By performing 15 replicates for each sample, the presence or absence of the paternal genotype was correctly predicted in 20/23 cases, with the remaining three cases being false negatives (Fig. 5, Table 1). Further analysis with an additional 25-replicate protocol eliminated 2 of the 3 false negative results. Moreover, two of the three false negative cases were performed using samples archived for more than 4 years and had been subjected to repeated freeze-thaw cycles.

[089] The approach of this invention takes advantage of the Poisson distribution of DNA fragments at single molecule concentration detected in replicates to further increase the sensitivity of mass-spectrometric analysis while retaining its high specificity. Using the described example we have shown that the approach allows the determination of the presence or absence of the paternally-inherited fetal mutation in maternal plasma covering the four most common β -thalassemia mutations in Southeast Asians.

[090] Three of the four analyzed mutations are point mutations and thus the results also demonstrate the feasibility of this method to detect even single nucleotide differences at very low fractional concentrations.

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[091] The ability to reliably analyze fetal DNA isolated from the maternal plasma offers great opportunity to practically provide non-invasive prenatal diagnosis. Our system using, for example, the MassARRAY approach is automatable with a throughput capability of analyzing more than 1200 samples per day with the about 15-replicate format, thus making the system practical for routine use.

[092] The present approach can be applied to at-risk pregnancies where the maternal and paternal mutations differ. However, the approach can be modified by incorporating the simultaneous detection of single nucleotide polymorphisms (SNPs) that are associated with either the mutated or normal paternal allele. The additional assessment of SNPs will also help to eliminate the false-negative results, as a diagnostic result is only regarded as valid if a paternal SNP allele is detected in at least one of the replicates.

[093] The MassARRAY system was originally designed for high-throughput SNP detection (Tang, K. *et al. Proc Natl Acad Sci U S A* **96**, 10016-10020 (1999)). The discrimination of paternal and maternal mutant alleles that share the same mutation can be achieved by the detection of SNPs that are linked particularly to the paternal mutation. The detection of SNPs associated with the paternal normal or healthy allele instead, also allows the positive exclusion of β -thalassemia (Chiu, R.W.K. *et al. Clin Chem* **48**, 778-780 (2002)). In summary, the analytical and prenatal diagnostic approach of the present invention presents a non-invasive prenatal diagnosis of autosomal recessive diseases including but not limited to the thalassemias, cystic fibrosis and congenital adrenal hyperplasia. This approach can also be applied to the other diagnostic applications of plasma DNA, such as the detection of tumor-derived point mutations in cancer patients (Anker, P. *et al. Gastroenterology* **112**, 1114-1120 (1997)) and donor-derived DNA in the plasma of transplant recipients (Lo, Y.M.D. *et al. Lancet* **351**, 1329-1330 (1998)).

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Example 2

[094] In the present study, we evaluated, and show the feasibility of, the use of MS for the discrimination of fetal point mutations in maternal plasma and developed an approach for the reliable exclusion of β -thalassemia mutations in maternal plasma. We further evaluated, and show the feasibility of, the approach for the noninvasive prenatal diagnosis of a mother and father sharing an identical β -thalassemia mutation, a concurrence previously perceived as a challenge for maternal plasma-based prenatal diagnosis for autosomal recessive diseases.

[095] In this example, mass spectrometric analysis of single nucleotide difference in circulating nucleic acids was applied to noninvasive or minimally invasive prenatal diagnosis.

[096] **Patient Recruitment and Sample Collection.** Twelve pregnancies at risk for β -thalassemia major were recruited with informed consent and institutional ethics approval from established prenatal diagnostic centers in Hong Kong, Thailand, Singapore, and Malaysia. Fifty pregnant women seeking second-trimester aneuploidy prenatal diagnosis with subsequent confirmation of a normal fetal karyotype also were recruited. Ten milliliters of maternal and paternal blood was collected into EDTA tubes before amniocentesis, chorionic villus sampling, and cordocentesis. Three milliliters of amniotic fluid also was collected from the normal pregnancies and stored at 4°C until analysis. Parental and fetal genotypes were determined according to established diagnostic practices (Ng, I. S., Ong, J. B., Tan, C. L. & Law, H. Y. (1994) *Hum. Genet.* **94**, 385-388; Sanguansermisri, T., Thanarattanakorn, P., Steger, H. F., Tongsong, T., Chanprapaph, P., Wanpirak, C., Siriwatanapa, P., Sirichotiyakul, S. & Flatz, G. (2001) *Hemoglobin* **25**, 19-27). Maternal plasma was harvested by a two-step centrifugation protocol comprised of 10-min centrifugation at 1,600 x g, followed by 10-min

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centrifugation at 16,000 x g (Chiu, R. W. K., Poon, L. L. M., Lau, T. K., Leung, T. N., Wong, E. M. C. & Lo, Y. M. D. (2001) *Clin. Chem.* **47**, 1607-1613). Maternal plasma DNA was extracted with the QIAamp Blood Kit (Qiagen, Valencia, CA) by following the "blood and body fluid protocol," according to the manufacturer's recommendations. To each column, 800 µl of plasma was applied and eluted into 50 µl of distilled deionized H₂O. The plasma DNA samples were stored at -20°C until analysis by a central laboratory.

[097] **Maternal Plasma Analysis.** Paternal allele detection in maternal plasma was performed by using the MassARRAY system (Sequenom). The MassARRAY system is a matrix-assisted laser desorption ionization/time-of-flight MS system designed for the detection of primer-extended PCR products (Tang, K., Fu, D. J., Julien, D., Braun, A., Cantor, C. R. & Koster, H. (1999) *Proc. Natl. Acad. Sci. USA* **96**, 10016-10020). The maternal plasma MS analyses were performed blindly without knowledge of the fetal genotype. Two analytical protocols were evaluated, including the standard Homogenous MassEXTEND protocol provided by Sequenom and a newly developed protocol, termed single allele base extension reaction (SABER) (Fig. 3). Both protocols involved PCR amplification of the paternally inherited fetal allele and the maternal background alleles from maternal plasma, followed by a base extension reaction before MS analysis. The SABER protocol involves a different base extension step, which is restricted to the allele of interest, and confers theoretical improvements in the detection sensitivity.

[098] **PCR Amplification.** All DNA oligonucleotides were purchased from Integrated DNA Technologies (Coralville, IA). HotStar *Taq* Polymerase (Qiagen) was used for all PCRs. Five microliters of plasma DNA was added to each 10-µl PCR. PCR primers (Fig. 6, Table 2) were used at a 200 nM final concentration. The PCR condition was 95°C for 15 min for hot start, followed by denaturing at 94°C for 20 sec, annealing at 56°C for 30 sec,

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extension at 72°C for 1 min for 45 cycles, and final incubation at 72°C for 3 min. Five microliters of PCR products was treated with shrimp alkaline phosphatase (Sequenom) for 20 min at 37°C to remove excess dNTPs, as described in Ding, C. & Cantor, C. R. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 7449-7453.

[099] **Standard Base Extension and SABER.** Thermosequenase (Sequenom) was used for the base extension reactions. In the standard protocol, conventional base extension was carried out whereby both alleles interrogated by the base extension primer were extended by adding a mixture of 2',3'-dideoxynucleoside triphosphates and dNTPs (Fig 6, Table 2 and Fig. 3). In contrast, primer extension in the SABER protocol was restricted to the fetal-specific allele of interest by the addition of a single species of dideoxynucleoside triphosphate without any dNTP (Fig. 6, Table 2 and Fig. 3). Five microliters of PCR products was used in 9- μ l reactions in both protocols. The reaction condition was 94°C for 2 min, followed by 94°C for 5 sec, 52°C for 5 sec, and 72°C for 5 sec for 40 cycles. All reactions were carried out in a GeneAmp PCR system 9700 thermal cycler (Applied Biosystems). The final base extension products were analyzed by MS as described in Ding, C. & Cantor, C. R. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 7449-7453. Briefly, the final base extension products were treated with the SpectroCLEAN (Sequenom) resin to remove salts in the reaction buffer. We dispensed \approx 10 nl of reaction solution onto a 384-format SpectroCHIP (Sequenom) prespotted with a matrix of 3-hydroxypicolinic acid by using a SpectroPoint (Sequenom) nanodispenser. A modified Biflex matrix-assisted laser desorption ionization/time-of-flight MS (Bruker, Billerica, MA) was used for data acquisitions from the SpectroCHIP. The expected molecular weights of all relevant peaks were calculated before the analysis and identified from the mass spectrum. All analyses were performed in triplicate.

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[0100] **Fetal-Specific Single-Nucleotide Polymorphism (SNP) Detection from Maternal Plasma.** The feasibility of using the MassARRAY system to discriminate and detect single-nucleotide differences between fetal and maternal DNA in maternal plasma was first assessed by the detection of paternally inherited SNPs. The maternal and fetal genotypes for 11 SNPs on chromosome 11p were determined in normal pregnancies by using maternal genomic DNA and amniotic fluid samples. The most informative SNP, rs2187610 (SNP database, www.ncbi.nlm.nih.gov), was selected for further analysis. This SNP is located 1.3 kb downstream of the *HBB* locus.

[0101] **Fetal-Specific β -Thalassemia Mutation Detection from Maternal Plasma.** MassARRAY assays (Fig. 6, Table 2) were designed for maternal plasma analysis of the four most common β -thalassemia mutations in Southeast Asia, CD 41/42 -CTTT, IVS2 654 (C \rightarrow T), nt -28 (A \rightarrow G), and CD 17 (A \rightarrow T) (Lau, Y. L., Chan, L. C., Chan, Y. Y., Ha, S. Y., Yeung, C. Y., Waye, J. S. & Chui, D. H. (1997) *N. Engl. J. Med.* **336**, 1298-1301; Liang, R., Liang, S., Jiang, N. H., Wen, X. J., Zhao, J. B., Nechtman, J. F., Stoming, T. A. & Huisman, T. H. (1994) *Br. J. Haematol.* **86**, 351-354). Paternal mutation detection in maternal plasma was determined by using both protocols. For each sample, the mutation-specific assay was selected according to the mutation that the father carried.

[0102] **Fetal Haplotype Detection from Maternal Plasma.** The parental genotypes at the SNP locus, rs2187610, were determined for the pregnancies at risk for β -thalassemia major. For parents who were found to be informative for the SNP, the linkage between the paternal *HBB* mutant with the SNP alleles at rs2187610 was determined. Haplotype analysis was determined by using a method on parental genomic DNA described in Ding, C. & Cantor, C. R. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 7449-7453. The ability to detect the

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paternal SNP linked to the mutant *HBB* allele in maternal plasma was determined by using the SABER protocol.

[0103] **Fetal-Specific SNP Allele Discrimination in Maternal Plasma.** The SNP rs2187610 is a C/G polymorphism. Among the 50 normal pregnancies, 16 pregnant women had the CC genotype. The fetal genotypes were CC and GC in 10 and 6 of these pregnancies, respectively. MassARRAY assays were designed to detect the paternally inherited fetal-specific *G* allele in maternal plasma (Fig. 6, Table 2). The presence or absence of the *G* allele in maternal plasma was concordant between the standard and SABER protocols, and these results were completely concordant with amniotic fluid analyses.

[0104] **Paternally Inherited β -Thalassemia Point Mutation Detection and Exclusion in Maternal Plasma.** Among the 12 recruited pregnancies at risk for β -thalassemia major, 11 pregnancies involved couples in which the father and mother carried different β -thalassemia mutations (Fig. 7, Table 3). Assays were designed to interrogate the four β -thalassemia mutations in maternal plasma, three of which were point mutations. The results are shown in (Fig. 7, Table 3). Detection of the paternal mutation in maternal plasma by using the SABER protocol was completely concordant with the fetal genotype determined by amniotic fluid, chorionic villus, or fetal blood analyses, whereas the standard protocol revealed two false-negative results (cases 5 and 9). Representative MS tracings for the analyses are shown in Fig. 4.

[0105] **Noninvasive Fetal Haplotyping.** SNP analysis for the at-risk pregnancies revealed three informative couples (cases 3, 11, and 12), including the parents sharing an identical β -thalassemia mutation, whereby the maternal and paternal SNP genotypes were nonidentical. Results of the haplotype analysis are shown in Fig. 8, Table 4. The paternal

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mutant allele was linked to the *G* allele at rs2187610 for the three cases. Maternal plasma analysis for the paternal *G* allele was completely concordant with the expected fetal genotype.

[0106] The reliable discrimination of subtle (e.g., single base) differences between fetal and maternal DNA in maternal plasma has hitherto been a technical challenge (Nasis, O., Thompson, S., Hong, T., Sherwood, M., Radcliffe, S., Jackson, L. & Otevre, T. (2004) *Clin. Chem.* **50**, 694-701). In this study, we took advantage of the analytical specificity conferred by a base extension reaction and the sensitivity of MS analysis. The SABER protocol is theoretically more sensitive than the standard protocol. First, in contrast to the standard protocol in which all relevant alleles are used as the templates for the base extension reaction, SABER involves the extension of a single nucleotide for the allele of interest only (Fig. 3). Thus, for fetal DNA analysis in maternal plasma, the SABER assays were designed so that the base extension is devoted only to the extension of the fetal-specific allele for the single discriminatory nucleotide from the maternal one. Furthermore, the matrix-assisted laser desorption ionization/time-of-flight MS has a dynamic range of ≈ 100 -fold. Because the paternal-specific fetal allele exists at ≈ 3 –6% in total maternal plasma DNA, its corresponding peak in the mass spectrum is often dwarfed by the background peak when analyzed by the standard protocol (Figs. 3 and 4). In contrast, the SABER method only extends the intended paternal-specific fetal allele so that the background allele peak is not produced, resulting in more robust detection (Figs. 3 and 4). The theoretical advantages of SABER over the standard method are realized in our analyses as evident by the false-negative results for the latter protocol.

[0107] The reliability of the SABER assays for single-nucleotide discrimination between circulating fetal and maternal DNA has been illustrated by the maternal plasma detection of fetal β -thalassemia point mutations and SNPs. The ability to robustly analyze fetal-specific

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SNPs in maternal plasma is a useful adjunct procedure for maternal-plasma fetal DNA analysis as a safeguard against the possibility of false-negative detection due to fetal DNA degradation, DNA extraction failures, or PCR allele dropout. Such a safeguard mechanism has been advocated by several workers in the routine performance of maternal plasma analysis for the noninvasive prenatal assessment of fetal rhesus D status (van der Schoot, C. E., Tax, G. H., Rijnders, R. J., de Haas, M. & Christiaens, G. C. (2003) *Transfusion Med. Rev.* **17**, 31-44; Zhong, X. Y., Holzgreve, W. & Hahn, S. (2001) *Swiss Med. Wkly.* **131**, 70-74; Avent, N. D., Finning, K. M., Martin, P. G. & Soothill, P. W. (2000) *Vox Sanguinis* **78**, 155-162). Initially, the detection of Y-chromosome sequences in maternal plasma had been adopted to confirm cases that tested negative for *RHD* (Finning, K. M., Martin, P. G., Soothill, P. W. & Avent, N. D. (2002) *Transfusion* **42**, 1079-1085; van der Schoot, C. E., Tax, G. H., Rijnders, R. J., de Haas, M. & Christiaens, G. C. (2003) *Transfusion Med. Rev.* **17**, 31-44). Because of the inherent restriction of Y-chromosome detection to only male fetuses, fetal-specific internal controls based on panels of insertion/deletion polymorphisms had been developed (Avent, N. D., Finning, K. M., Martin, P. G. & Soothill, P. W. (2000) *Vox Sanguinis* **78**, 155-162). The adoption of the insertion/deletion panel reflects the lack of robust methods for fetal SNP detection in the past. Hence, with the availability of a reliable MS method for fetal SNP detection in maternal plasma, the number of potential gender-independent internal control targets for circulating fetal DNA detection has increased substantially.

[0108] A more important implication of the ability to analyze circulating fetal SNPs lies in its immediate relevance to fetal haplotype analysis from maternal plasma. Noninvasive fetal haplotyping could be achieved by means of analyzing polymorphisms linked to a mutated locus. As demonstrated in case 12, haplotype analysis between the *HBB* locus and a

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linked polymorphism allowed the noninvasive prenatal exclusion of β -thalassemia major, despite the presence of the same *HBB* mutation in both parents, which had previously not been believed practical. (Chiu, R. W. K., Lau, T. K., Leung, T. N., Chow, K. C. K., Chui, D. H. K. & Lo, Y. M. D. (2002) *Lancet* **360**, 998-1000). The haplotype approach is also applicable to maternal plasma detection of a fetal SNP allele linked to the paternal nonmutant allele. The positive detection of such an allele would allow for the positive prenatal exclusion of β -thalassemia major noninvasively (Chiu, R. W. K., Lau, T. K., Cheung, P. T., Gong, Z. Q., Leung, T. N. & Lo, Y. M. D. (2002) *Clin. Chem.* **48**, 778-780; Bianchi, D. W. (2002) *Clin. Chem.* **48**, 689-690).

[0109] Additional SNP markers surrounding the *HBB* locus can be easily assessed by a person skilled in the art. For example, an SNP panel could be assembled so that the noninvasive prenatal diagnosis could be applied to a larger proportion of pregnancies at risk for β -thalassemia. The four mutations investigated in this study account for 90% of all β -thalassemia mutations in Southeast Asia (Lau, Y. L., Chan, L. C., Chan, Y. Y., Ha, S. Y., Yeung, C. Y., Waye, J. S. & Chui, D. H. (1997) *N. Engl. J. Med.* **336**, 1298-1301; Liang, R., Liang, S., Jiang, N. H., Wen, X. J., Zhao, J. B., Nechtman, J. F., Stoming, T. A. & Huisman, T. H. (1994) *Br. J. Haematol.* **86**, 351-354). The present approach can easily be applied to all pregnancies in which the father is a carrier of one of the four mutations and thus has much potential for routine adoption. An invasive prenatal diagnostic procedure could be avoided in 50% of these pregnancies in which the lack of inheritance of the paternal mutation by the fetus is confirmed by maternal plasma analysis.

[0110] This study shows technological advancements in circulating fetal DNA analysis, and that we have developed robust system for single-nucleotide discrimination among circulating DNA species. The MassARRAY approach is automatable with a capacity to

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analyze >2,000 samples per day in triplicate, thus making the system practical for routine use. The MS system is potentially to many other areas of fetal DNA detection, namely the prenatal diagnosis of other single-gene disorders and the quantification of fetal DNA in maternal plasma (Ding, C. & Cantor, C. R. (2003) *Proc. Natl. Acad. Sci. USA* **100**, 3059-3064).

Quantitative aberrations in circulating fetal DNA concentrations have been demonstrated for fetal chromosomal aneuploidies (Lo, Y. M. D., Lau, T. K., Zhang, J., Leung, T. N., Chang, A. M., Hjelm, N. M., Elmes, R. S. & Bianchi, D. W. (1999) *Clin. Chem.* **45**, 1747-1751; Zhong, X. Y., Burk, M. R., Troeger, C., Jackson, L. R., Holzgreve, W. & Hahn, S. (2000) *Prenatal Diagn.* **20**, 795-798), preeclampsia (Lo, Y. M. D., Leung, T. N., Tein, M. S., Sargent, I. L., Zhang, J., Lau, T. K., Haines, C. J. & Redman, C. W. (1999) *Clin. Chem.* **45**, 184-188; Zhong, X. Y., Laivuori, H., Livingston, J. C., Ylikorkala, O., Sibai, B. M., Holzgreve, W. & Hahn, S. (2001) *Am. J. Obstet. Gynecol.* **184**, 414-419), preterm labor (Leung, T. N., Zhang, J., Lau, T. K., Hjelm, N. M. & Lo, Y. M. D. (1998) *Lancet* **352**, 1904-1905), and many other pregnancy-associated complications. Quantitative analysis of circulating fetal DNA has been reliant on the detection of Y-chromosome sequences because of the lack of gender-independent fetal-specific markers. However, this hurdle can be overcome by the adoption of MS quantification of fetal SNPs in maternal plasma. Both the MS approach and the gender-independent fetal SNP assays can be extended to the study of fetal DNA in other maternal bodily fluids such as urine (Botezatu, I., Serdyuk, O., Potapova, G., Shelepov, V., Alechina, R., Molyaka, Y., Ananev, V., Bazin, I., Garin, A., Narimanov, M., *et al.* (2000) *Clin. Chem.* **46**, 1078-1084) and cerebrospinal fluid (Angert, R. M., Leshane, E. S., Yarnell, R. W., Johnson, K. L. & Bianchi, D. W. (2004) *Am. J. Obstet. Gynecol.* **190**, 1087-1090) or the phenomenon of cellular microchimerism (Bianchi, D. W. & Romero, R. (2003) *J. Maternal Fetal Neonatal Med.* **14**, 123-129; Nelson, J. L. (2001) *Lancet* **358**, 2011-2012; Lo, Y. M. D.,

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Lo, E. S., Watson, N., Noakes, L., Sargent, I. L., Thilaganathan, B. & Wainscoat, J. S. (1996) *Blood* **88**, 4390-4395), all of which also have been previously studied by means of the detection of Y-chromosome sequences (Lo, Y. M. D., Patel, P., Wainscoat, J. S., Sampietro, M., Gillmer, M. D. & Fleming, K. A. (1989) *Lancet* **2**, 1363-1365; Lo, Y. M. D., Patel, P., Wainscoat, J. S. & Fleming, K. A. (1990) *Lancet* **335**, 724 (lett.)).

[0111] In addition to fetal DNA sequences, the MS SABER approach can also be extended to other areas of circulating nucleic acid analysis, including circulating tumor-specific DNA, such as Epstein-Barr virus DNA in nasopharyngeal carcinoma patients (Lo, Y. M. D., Chan, L. Y. S., Lo, K. W., Leung, S. F., Zhang, J., Chan, A. T. C., Lee, J. C., Hjelm, N. M., Johnson, P. J. & Huang, D. P. (1999) *Cancer Res.* **59**, 1188-1191), *KRAS* point mutations (Anker, P., Lefort, F., Vasioukhin, V., Lyautey, J., Lederrey, C., Chen, X. Q., Stroun, M., Mulcahy, H. E. & Farthing, M. J. (1997) *Gastroenterology* **112**, 1114-1120; Sorenson, G. D. (2000) *Ann. N.Y. Acad. Sci.* **906**, 13-16), and donor-specific DNA in transplant recipients (Lo, Y. M. D., Tein, M. S., Pang, C. C., Yeung, C. K., Tong, K. L. & Hjelm, N. M. (1998) *Lancet* **351**, 1329-1330). Therefore, we believe that MS will play an increasingly important role in the future research and application of circulating nucleic acids.

[0112] All references cited herein and throughout the specification are incorporated herein by reference in their entirety.

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CLAIMS

We claim:

1. A method of determining a single gene disorder in a fetus from a plasma, whole blood, or serum sample of a pregnant mother, the method comprising:
 - a) analyzing nucleic acid samples isolated from the pregnant mother and a father for a disease-causing mutation for a single gene disorder or a single nucleotide polymorphism associated with a disease-causing mutation;
 - b) isolating nucleic acid from blood, plasma, or serum of the pregnant mother;
 - c) determining a fetal genotype from the nucleic acid isolated in step b) using primers corresponding to a disease-causing mutation allele or mutation-associated allele containing a single nucleotide polymorphism identified in the nucleic acid from the father in step a) and differentially amplifying the alleles from the isolated nucleic acid sample of step b) in replicates and detecting the amplified products, wherein a detection of a paternal mutation in any of the replicate sample is indicative of the presence of the single-gene disorder in the fetus.
2. The method of claim 1, wherein the single gene disease is an autosomal recessive disease.
3. The method of claim 2, wherein the autosomal recessive disease is selected from beta thalassemia, cystic fibrosis and congenital adrenal hyperplasia.
4. The method of claim 3, wherein the disease is beta thalassemia caused by mutations selected from the group consisting of CD 41/42 -CTTT; IVS2 654 (C->T); nucleotide -28 (A->G); and CD 17 (A->T).
5. The method of claim 1, number of replicates is 10-100.

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6. The method of claim 1, wherein the number of replicates is 15-25.
7. The method of claim 1, wherein the differential amplification is followed by MassARRAY system.
8. A method of detecting a genetic disease or characteristic in a fetus using maternally blood, plasma, serum, the method comprising:
 - a) selecting one or more single nucleotide polymorphisms (SNP) which are not disease-causing polymorphisms and which are associated either with a paternal disease-causing allele or with a paternal healthy allele and which SNP differs between the maternal and the paternal genotype;
 - b) determining the fetal genotype from a sample DNA isolated from the blood, plasma, serum of the pregnant mother, wherein the determination is performed using primers corresponding to both the selected SNP and the disease-causing mutation and performing an SNP and disease causing mutation-specific or disease causing mutation allele-specific enhancement and analysis in several replicates using said primers, wherein detection of the SNP associated with the paternal allele in any of the replicate samples is indicative of the presence of the paternal allele inherited by the fetus and the detection of the paternal disease-causing mutation in any of the replicate samples indicates detection of the genetic disease inherited by the fetus or the detection of the SNP associated with the healthy paternal allele excludes inheritance of the genetic disease by the fetus.
9. A method of detecting a paternally inherited nucleic acid region in a fetus comprising isolating nucleic acids from blood, plasma or serum of a pregnant mother, amplifying the nucleic acids in the sample with PCR primers designed

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to anneal to regions flanking a genetic locus which carries a difference between the maternal and the paternal nucleic acid, performing a base extension reaction; and analyzing products of the base extension reaction, wherein the presence of the base extension product corresponding to the nucleic acid present in the paternal nucleic acid indicates that the fetus carries the paternally inherited nucleic acid region in the genetic locus.

10. The method of claim 9, wherein the base extension reaction is performed using single allele base extension reaction.
11. The method of claim 9 and 10, wherein analyzing products of the base extension reaction is performed using mass spectrometry.

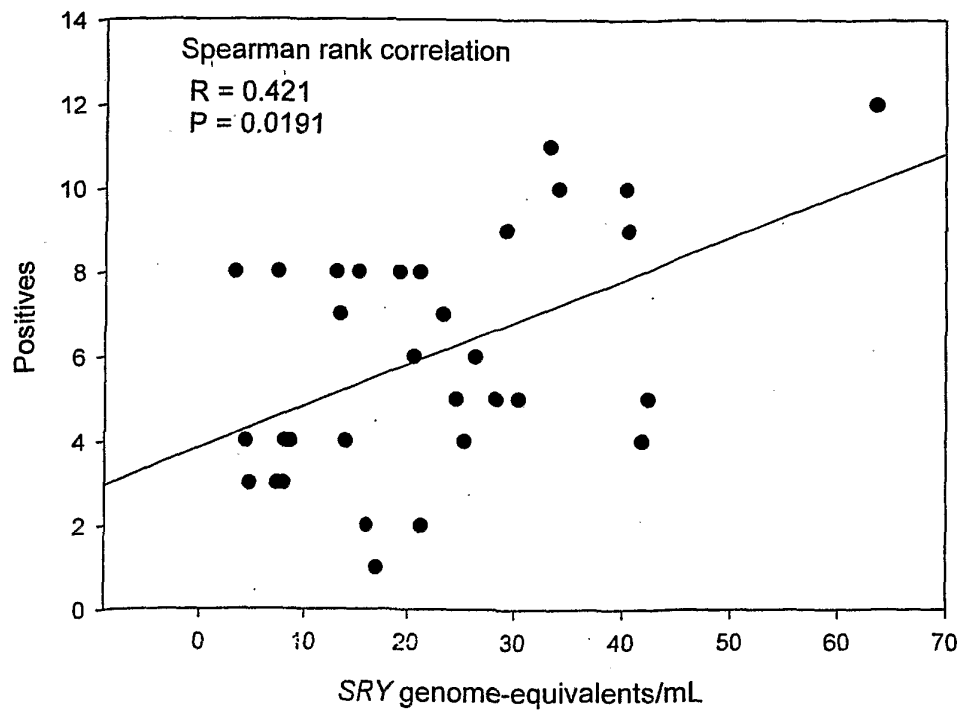
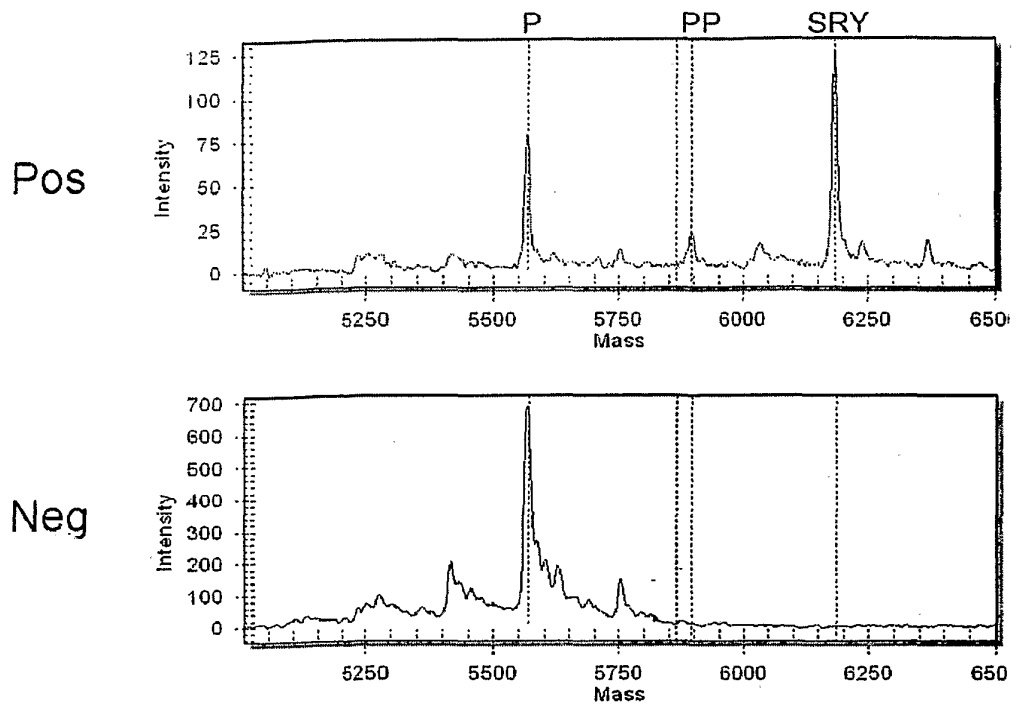
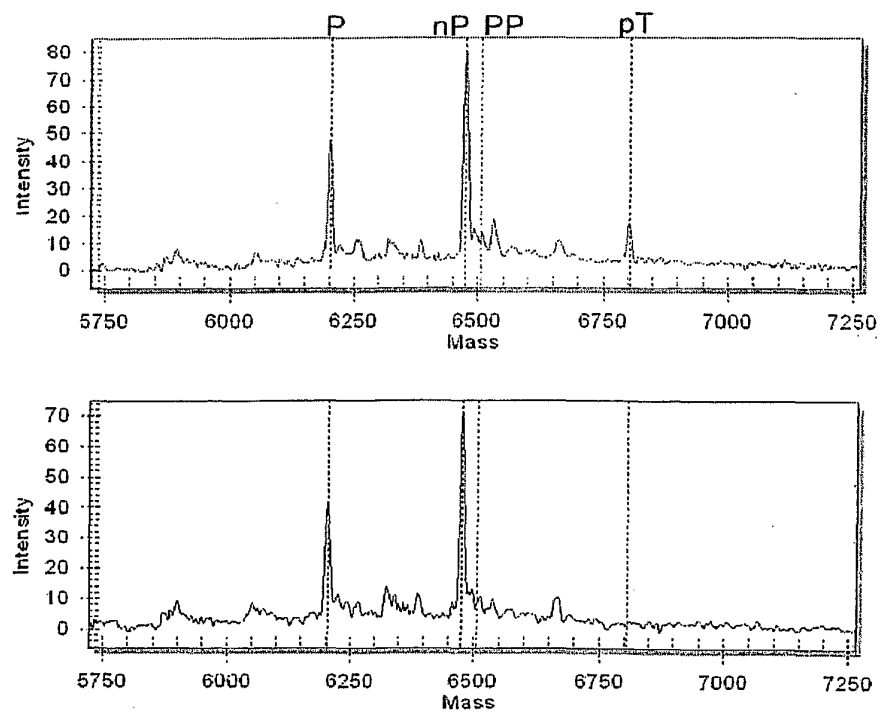


FIGURE 1

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FIG. 2A. SRY**FIG 2B. Thalassemia IVS2 654****FIGURE 2**

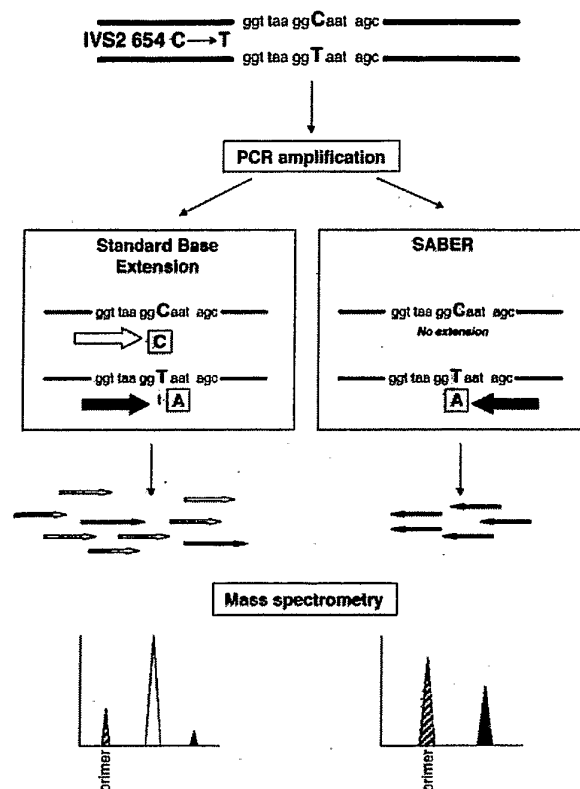


FIGURE 3

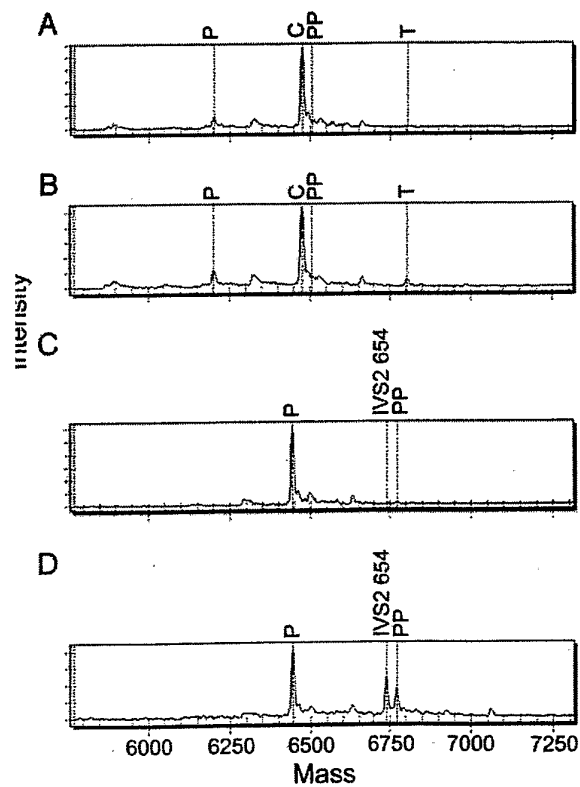


FIGURE 4

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Case	CD 41/42 (-CTTT)	HBB Mutation ^a IVS2 654 (C→T)	nt -28 (A→G)	CD 17 (A→T)	Fetal genotype ^b	No. of positive replicates ^c	Gestational age (weeks)
1	F				F/M	6	14
2	M	F			F/*	4	12
3	M	F			F/*	4	16
4	F	M			*/*	0	10
5		F		M	*/*	0	18
6	F			M	*/*	0	21
7	M	F			F/*	3	17
8	F	M			F/*	7	13
9	F		M		F/M	3	18
10	M	F			*/*	0	11
11	M		F		F/M	7	16
12	M			F	*/*	0	22
13	F	M			*/M	0	12
14	F			M	F/M	3	18
15	M	F			F/M	0	8
16	F	M			F/M	0	12
17	F				*/M	0	12
18	F		M		F/M	2	19
19			M	F	F/*	6	17
20				F	F/*	0	19
21				F	F/*	3	19
22				F	F/M	0	19
23	F			M	*/*	0	19
					F/*	10	20

FIGURE 5

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Mutation	CD 41/42 (-CTTT)	IVS2 654 (C → T)	nt -28 (A → G)	CD 17 (A → T)	rs2187610
PCR primer 1	5'-ACGTTGGATG- AACAGCATCA- GGAGTGGAC-3'	5'-ACGTTGGATG- TAACAGTGAT- AATTCTGGG-3'	5'-ACGTTGGATG- TAGGGTTGGC- CAATCTACTC-3'	5'-ACGTTGGATG- TCACCACCAA- CTTCATCCAC-3'	5'-ACGTTGGATG- ATGCCATTTC- ATGGTTACC-3'
PCR primer 2	5'-ACGTTGGATG- CTATTTCCCA- CCCTTAGGC-3'	5'-ACGTTGGATGG- AAACCTCTTAC- ATCAGTTAC-3'	5'-ACGTTGGATG- AGCAATAGAT- GGCTCTGCCC-3'	5'-ACGTTGGATG- TCAAACAGAC- ACCATGGTGC-3'	5'-ACGTTGGATG- GAAGTGAGGC- TACATCAAAC-3'
Standard protocol					
Extension primer	5'-GATCCCCAAA- GGACTCAA-3'	5'-TGATAATTTCT- GGGTAAAGG-3'	5'-AGCCAGGGCT- GGGCATA-3'	5'-TTCATCCACGT- TCACCT-3'	5'-ACCTTTCATTG- TTCATTGTTTT-3'
Terminator mix*	CGT	AC	AC	CGT	ACT
Expected molecular weight of extended nonmutant allele	6,088	6,475	5,558	5,345	7,225 (G allele)
Expected molecular weight of extended mutant allele	5,735	6,804	5,887	5,683	7,569 (C allele)
SABER protocol					
Extension primer	5'-GATCCCCAAA- GGACTCAA-3'	5'-ATATGCAGAAA- TATTGCTATT-3'	5'-GATGGCTCTG- CCCTGACTT-3'	5'-TTCATGCCCT- GTGGGGC-3'	5'-ACCTTTCATTG- TTCATTGTTTT-3'
Terminator	ddCTP	ddATP	ddCTP	ddTTP	ddCTP
Expected molecular weight of nonextended primer	5,462	6,443	5,771	5,193	6,952
Expected molecular weight of extended allele	5,735	6,741	6,044	5,482	7,225 (G allele)

FIGURE 6

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Case	Genotype for SNP rs2187610		Paternal haplotype analysis [†]		Maternal plasma SABER analysis		
	Mother	Father	<i>HBB</i> mutant allele	<i>HBB</i> wild- type allele	SNP G allele	Paternal <i>HBB</i> mutation	Fetal <i>HBB</i> genotype [†]
3	CC	GC	G	C	Neg	Neg	*/*
11	CC	GC	G	C	Pos	Pos	F/*
12	CC	GC	G	C	Neg	N.A.	*/*

FIGURE 7

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Case	<i>HBB</i> mutation				Maternal plasma analysis		Fetal genotype [†]	Weeks gestation
	CD 41/42 (-CTTT)	IVS2 654 (C → T)	nt — 28 (A → G)	CD 17 (A → T)	Standard protocol	SABER		
1	F	M	—	—	Neg	Neg	*/*	11
2	—	F	—	M	Neg	Neg	*/*	18
3	F	—	—	M	Neg	Neg	*/*	21
4	M	F	—	—	Pos	Pos	F/*	18
5	M	—	F	—	Neg	Pos	F/M	17
6	F	M	—	—	Pos	Pos	F/*	11
7	F	M	—	—	Pos	Pos	F/*	14
8	F	—	—	—	Neg	Neg	*/*	7
9	—	—	—	F	Neg	Pos	F/*	12
10	M	F	—	—	Neg	Neg	*/*	17
11	F	—	—	—	Pos	Pos	F/*	20
12	M & F	—	—	—	N.A.	N.A.	*/*	18

FIGURE 8

Exhibit 18

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
2 October 2008 (02.10.2008)

PCT

(10) International Publication Number
WO 2008/118988 A1(51) International Patent Classification:
CI2Q 1/68 (2006.01) *CI2P 19/34* (2006.01)(74) Agents: **GRANT, Bruce, D.** et al.; Grant Anderson LLP,
C/O Portfoliop, P.O. Box 52050, Minneapolis, MN 55402
(US).(21) International Application Number:
PCT/US2008/058317(81) Designated States (*unless otherwise indicated, for every
kind of national protection available*): AE, AG, AL, AM,
AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA,
CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE,
EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID,
IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC,
LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN,
MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH,
PL, PT, RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, SV,
SY, TJ, TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN,
ZA, ZM, ZW.

(22) International Filing Date: 26 March 2008 (26.03.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:
60/908,167 26 March 2007 (26.03.2007) US(71) Applicant (*for all designated States except US*): **SE-
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(US). **VAN DEN BOOM, Dirk, J.** [DE/US]; 638 Bonair
Way, Unit A, La Jolla, CA 92037 (US).(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),
European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL,
NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG,
CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).**Published:**— *with international search report*

(54) Title: RESTRICTION ENDONUCLEASE ENHANCED POLYMORPHIC SEQUENCE DETECTION

(57) Abstract: Provided is an improved method for the detection of specific polymorphic alleles in a mixed DNA population. The method comprises enriching the relative percentage of a given polymorphic allele that is exponentially amplifiable by PCR. Also provided are methods for selectively enriching target nucleic acid, for example, fetal nucleic acid in a maternal sample. In the case of detecting fetal nucleic acid in a maternal sample, a restriction enzyme is introduced that can discriminate between the alleles of a polymorphic site. Preferably, the maternal allele is digested and nucleic acid comprising the paternal allele is relatively enriched.

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RESTRICTION ENDONUCLEASE ENHANCED POLYMORPHIC SEQUENCE DETECTION

FIELD OF THE INVENTION

Provided herein are methods for detecting specific alleles in a mixed nucleic acid sample.

5 The methods can be used to detect the presence or absence of fetal nucleic acid in a maternal sample.

BACKGROUND

10 The analysis of circulating nucleic acids has revealed applications in the non-invasive diagnosis, monitoring, and prognostication of many clinical conditions. For example, for prenatal applications, circulating fetal-specific sequences have been detected and constitute a fraction of the total DNA in maternal plasma. The diagnostic reliability of circulating DNA analysis depends on the fractional concentration of the targeted sequence, the analytical sensitivity, and the specificity. The robust discrimination of sequence differences (e.g., single-nucleotide
15 polymorphisms, or SNPs) between circulating DNA species is technically challenging and demands the adoption of highly sensitive and specific analytical methods.

Current techniques to detect sequence differences in a DNA sample include allele-specific PCR, restriction digest and Southern blot hybridization, restriction endonuclease-mediated selective-PCR (REMS-PCR), and competitive PCR methods involving the use of fluorescent
20 detection probes. The currently available techniques present several disadvantages. For allele-specific PCR, it is often difficult to design assays with a high degree of allele specificity (Nasis et al. *Clin Chem.* 2004 Apr;50(4):694-701). Restriction digest/Southern blot methods require higher amounts of DNA template than the method provided herein, and lack the sensitivity to detect polymorphic sequences comprising a low relative proportion of total DNA. Restriction
25 endonuclease-mediated selective-PCR (REMS-PCR) has the drawback of requiring a thermostable restriction enzyme that cleaves the wild-type allele. REMS-PCR is described in US Patent No. 6,261,768, which is hereby incorporated by reference. Use of the technique may not always be possible, and this requirement limits the general utility of the REMS-PCR approach. Competitive PCR lacks the sensitivity to detect polymorphic sequences comprising a low relative proportion
30 (<5%) of total DNA. Competitive PCR with allele-specific fluorescent probes lacks the ability to multiplex assays higher than 2-3 assays in a single tube format.

In addition, similar methods utilizing methylation differences between DNA species (for example, US Patent Application Publication No. 20070059707, entitled, "Methods for prenatal diagnosis of chromosomal abnormalities", which is hereby incorporated by reference) are not
35 effective at low copy numbers of genomic DNA.

SUMMARY

The invention in part provides sequence-specific cleavage of nucleic acid to selectively enrich for a particular target nucleic acid. Polymorphic loci are chosen such that only one allele at the polymorphic locus is cleaved by a given cleavage agent, such as a restriction endonuclease.

5 Oligonucleotide primer pairs designed to flank the polymorphism allow amplification of the polymorphic region, or amplicon, by amplification (*e.g.*, PCR). Prior to or during amplification, nucleic acid samples are incubated with the given restriction endonuclease. In a preferred embodiment, the cleavage agent is introduced prior to amplification. This embodiment results in cleavage of the polymorphic allele or sequence comprising the polymorphic allele that is
10 recognized by the restriction endonuclease, if this allele is present. Cleavage of any template nucleic acid within the amplicon sequence (*i.e.*, between primer pairs) prevents PCR amplification of this template. Therefore, if only one allele of a polymorphism is recognized by the cleavage agent and the corresponding nucleic acid sequence is cleaved by the restriction endonuclease, the relative percentage of the amplifiable alternate polymorphic allele is increased in a manner
15 dependent on the efficiency and specificity of the restriction endonuclease activity. After amplification, the amplified polymorphic alleles can be genotyped or otherwise detected or discriminated by any method known in the art (*e.g.*, using Sequenom's MassARRAY® technology or by RT-PCR).

In one embodiment, the invention in part provides a method for detecting the presence or
20 absence of a target allele at a polymorphic locus in a sample, wherein the sample contains nucleic acid, which comprises: cleaving a nucleic acid comprising a non-target allele at or near the polymorphic locus with a cleavage agent that recognizes and cleaves a non-target allele, but not the target allele; amplifying uncleaved nucleic acid but not cleaved nucleic acid; and analyzing the amplification products from the previous step to determine the presence or absence of the target
25 allele. In a related embodiment, the method also comprises first obtaining a sample suspected of comprising nucleic acid with target and non-target alleles. In a preferred embodiment, the method is used to distinguish between two individuals, for example, between a mother and a fetus, wherein the sample comprises both maternal and fetal nucleic acid. Optionally, the method may be used to quantify the target nucleic acid relative to the non-target nucleic acid.

30 The invention in part provides methods for enriching for target nucleic acid, comprising cleaving nucleic acid comprising a non-target allele with a restriction endonuclease that recognizes the nucleic acid comprising the non-target allele but not the target allele; and amplifying uncleaved nucleic acid but not cleaved nucleic acid, wherein the uncleaved, amplified nucleic acid represents enriched target nucleic acid relative to non-target nucleic acid. In one embodiment, the methods
35 may be utilized to determine the presence or absence of target nucleic acid in a background of

non-target nucleic acid. In a related embodiment, the amplification products can be analyzed to diagnose, monitor or prognose a clinical condition. Likewise, the amplification products can be analyzed to assist in the diagnosis, prognosis or monitoring of a clinical condition or chromosomal abnormality. Nucleic acid may be selected such that it comprises an allele having a polymorphic site that is susceptible to selective digestion by a cleavage agent, for example.

The methods are useful for analyzing nucleic acid including, but not limited to, DNA, RNA, mRNA, oligonucleosomal, mitochondrial, epigenetically-modified, single-stranded, double-stranded, circular, plasmid, cosmid, yeast artificial chromosomes, artificial or man-made DNA, including unique DNA sequences, and DNA that has been reverse transcribed from an RNA sample, such as cDNA, and combinations thereof. In one embodiment, the method is used to detect or selectively enrich RNA.

The nucleic acid may also be characterized as target nucleic acid or non-target nucleic acid, wherein target nucleic acid comprises the target allele and non-target nucleic acid comprises the non-target allele. In one embodiment, the target nucleic acid comprises the paternal allele and the non-target nucleic acid comprises the maternal allele. In certain embodiments, the nucleic acid is cell-free nucleic acid or partially cell-free nucleic acid. In certain embodiments, the target nucleic acid is apoptotic or partially apoptotic. In certain embodiments, the target nucleic acid is less than 2000, 1200, 1100, 1000, 900, 800, 700, 600, 500, 400, 300, 200, 100, 80, 70, 60, 50, 40 or less base pairs in length.

The methods may be used to detect target nucleic acid in a biological sample. Preferably, the biological sample is from an animal, preferably a human. In a related embodiment, the biological sample is selected from the group of whole blood, serum, plasma, umbilical cord blood, chorionic villi, amniotic fluid, cerebrospinal fluid, spinal fluid, lavage fluid, biopsy sample, urine, feces, sputum, saliva, nasal mucous, prostate fluid, semen, lymphatic fluid, bile, tears, sweat, breast milk, breast fluid, embryonic cells and fetal cells, and mixture thereof. In one embodiment, the sample is from a crime scene (e.g., used for forensic analysis). In certain embodiments, the biological sample is obtained through non-invasive means, for example, a blood draw from a pregnant female. In another preferred embodiment, the biological sample is cell-free. In certain embodiments, the sample is a previously isolated sample of nucleic acids.

In one embodiment, the invention in part provides a method for detecting the presence or absence of fetal nucleic acid in a maternal sample, wherein the sample contains nucleic acid, which comprises: cleaving nucleic acid comprising a maternal allele with a restriction endonuclease that recognizes and cleaves the nucleic acid comprising the maternal allele but not the paternal allele; amplifying uncleaved nucleic acid but not cleaved nucleic acid; and analyzing the amplification products from the previous step to determine the presence or absence of fetal

nucleic acid. In certain embodiments, the sample comprises a mixture of nucleic acids. For example, the mixture may comprise nucleic acid from different species or from different individuals. In one embodiment, the sample is from a pregnant female. Samples can be collected from human females at 1-4, 4-8, 8-12, 12-16, 16-20, 20-24, 24-28, 28-32, 32-36, 36-40, or 40-44 weeks of fetal
5 gestation, and preferably between 5-28 weeks of fetal gestation. In certain embodiments, the methods may be used to detect the presence or absence of fetal Y-chromosome nucleic acid, thereby determining the sex of the fetus.

In certain embodiments, the target nucleic acid comprises a paternal allele. In certain embodiments, the mother is homozygous at the polymorphic site and the fetus is heterozygous at
10 the polymorphic site. In the case when the mother is homozygous at the polymorphic site and the fetus is heterozygous at the polymorphic site, the polymorphic site is considered informative (see Figure 5A for examples of informative and non-informative cases). In a related embodiment, the maternal genotype is determined in conjunction with the methods provided herein. In a related
15 embodiment, the mother is first genotyped (for example, using peripheral blood mononuclear cells (PBMC) from a maternal whole blood sample) to determine the non-target allele that will be recognized and cleaved by the cleavage agent. When the method is used for forensic purposes, the victim may be first genotyped to determine the non-target allele that will be recognized and
20 cleaved by the cleavage agent. Likewise, when used for organ transplant-related applications, the transplant recipient may be first genotyped to determine the non-target allele that will be recognized and cleaved by the cleavage agent.

In some embodiments, the sample contains nucleic acid from two different individuals. Such instances include, but are not limited to, organ transplant recipients, transfusion recipients, and forensic applications.

In certain embodiments, the sample is from an individual suspected of suffering from a
25 disease, and the non-target allele is a wild-type allele that is selectively cleaved in order to enrich for a disease-related point mutation. In a related embodiment, the disease is cancer. The ras proto-oncogenes, K-ras, N-ras, and H-ras, and the p53 tumour suppressor gene are examples of genes which are frequently mutated in human cancers. Specific mutations in these genes leads to activation or increased transforming potential.

The invention in part provides methods useful for detecting rare alleles or low copy number
30 alleles. In one embodiment, the target allele is undetectable by conventional or unmodified genotyping methods if the non-target allele is not selectively cleaved. In a related embodiment, the target allele is not detectable unless it is selectively enriched, for example, by the methods provided herein. In certain embodiments, the target allele concentration (e.g., allele concentration
35 in a sample) is less than 0.5%, 1%, 2%, 3%, 4%, 5%, 6%, 7%, 8%, 9%, 10% 20%, 25%, 30%

relative to the non-target allele concentration. In certain embodiments, the target nucleic acid number is less than 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 55, 60, 70, 80, 90, 100, 150, 200, 250, 300, 400, 500, 600, 700, 800, 900 or 1000. In certain embodiments, the target allele is a mutation, and the non-target allele is the wild-type allele. In a
5 related embodiment, the target allele may be either a somatic or germline mutation. In certain embodiments, another allele or sequence identifier in the same amplicon as the polymorphic locus may be detected. For example, a sequence comprising a target allele may be selectively enriched using the methods provided herein, and another sequence identifier may be detected by any method known in the art.

10 In some embodiments, there are no other polymorphic loci within the amplicon that may be recognized by the cleavage agent.

In certain embodiments, the method optionally comprises first isolating nucleic acid from the sample. DNA isolation from blood, plasma, or serum of the pregnant mother can be performed using any method known to one skilled in the art. Any standard DNA isolation technique can be
15 used to isolate the fetal DNA and the maternal DNA including, but not limited to, QIAamp DNA Blood Midi Kit supplied by QIAGEN. Other standard methods of DNA isolation are described, for example, in (Sambrook et al., *Molecular Biology: A laboratory Approach*, Cold Spring Harbor, N. Y. 1989; Ausubel, et al., *Current protocols in Molecular Biology*, Greene Publishing, Y, 1995). A preferred method for isolation of plasma DNA is described in Chiu et al., 2001, *Clin. Chem.* 47: 1607-1613, which is herein incorporated by reference in its entirety. Other suitable methods are
20 provided in Example 2 of PCT International Application Publication Number 2007/028155, filed on September 1, 2006.

Methods described herein allow for the use of any cleavage agent capable of distinguishing between two different sequences, and cleaving within the amplicon sequence, thereby preventing
25 amplification of the cleaved sequence. The difference between the sequences may be the result of different alleles at one or more polymorphic sites within the sequence. In another example, the difference between the sequences may be the result of two homologous sequences, for example, between paralogous genes or between highly homologous genes such as the RhD gene, which encodes the D polypeptide, and the RHCE gene, which encodes the CcEe polypeptide. An
30 example of a cleavage agent is a restriction enzyme, also referred to as a restriction endonuclease. Multiple restriction endonucleases (available from various vendors) may be selected that correspond to appropriate sequence differences. In a preferred embodiment, the restriction enzyme is HpyCH4V. In another preferred embodiment restriction enzyme Tsp509I. In certain embodiments, a step is added to end the cleaving activity of the cleavage agent, for
35 example, by introducing a protease and/or high temperature prior to amplification.

The restriction endonuclease may be added prior to or during amplification. In one embodiment, the restriction endonuclease is added less than 5 minutes, 5 minutes, 10 minutes, 20 minutes, 30 minutes, 40 minutes, 50 minutes, 60 minutes, 90 minutes or 120 or more minutes before amplification. Incubation time may be shortened if additional units of restriction enzyme are added to the reaction. Conversely, longer incubation times are often used to allow a reaction to proceed to completion with fewer units of enzyme. This is contingent on how long a particular enzyme can survive (maintain activity) in a reaction. Some enzymes survive for long periods (> 16 hours) while others survive only an hour or less in a reaction. In certain embodiments, the restriction enzyme digests greater than 40%, 50%, 60%, 70%, 80%, 90%, 91%, 92%, 93%, 94%, 95%, 96%, 97%, 98% or 99% of the non-target nucleic acid. However, if digestion of non-target nucleic acid of less than 40% allows for useful enrichment of target nucleic acid, it is within the scope of the invention. In certain embodiments, the restriction enzyme digests substantially all of the non-target nucleic acid. In certain embodiments, when the restriction endonuclease is added during amplification, the restriction endonuclease is a thermostable restriction endonuclease which retains activity during thermocycling. Examples of thermostable endonucleases include, but are not limited to, Bst NI, Bsl I, Tru 9I and Tsp 509 I. In certain embodiments, the cleavage agent is not thermostable (non-thermostable), especially when it is preferred that the digestion occurs prior to the amplification step. In a preferred embodiment, the cleavage agent is not thermostable and digestion of the non-target nucleic acid occurs prior to the amplification step. In certain embodiments, a step is introduced to prevent or to reduce digestion during the amplification step.

In one embodiment, the units of restriction enzyme added to the sample is 0.10, 0.25, 0.50, 0.75, 1.0, 2.0 or more. Note that DNA substrates are digested at varying rates, therefore, the actual number of units required for a complete or substantially complete digestion may vary from assay to assay.

In certain embodiments, only one restriction endonuclease is used to digest one or more non-target alleles in a single reaction. For example, a multiplexed assay may be designed wherein a single restriction endonuclease performs multiple (*e.g.*, greater than 5, 10, 15, 20, 25, 50, 100) digestions across the genome. In certain embodiments, more than one restriction endonuclease (*e.g.*, greater than or equal to 2, 3, 4, 5, 6, 7, 8, 9, 10) is used to make multiple (*e.g.*, greater than 5, 10, 15, 20, 25, 50, 100) digestions across the genome.

Amplification may be performed after or during the cleavage of the non-target allele, and prior to the detection of the target allele. In a preferred embodiment, amplification is performed after cleavage of the non-target allele. Amplification can be performed by any method known in the art, including but not limited to polymerase chain reaction (PCR), ligase chain reaction, transcription-based amplification, restriction amplification, or rolling circle amplification, using

primers that anneal to the selected fetal DNA regions. In the case that amplification requires thermocycling, cycling greater than 90° C may be performed such that the cleavage agent is inactivated. Oligonucleotide primers are selected such that they anneal to the sequence to be amplified. In one embodiment, primers are designed such that one or both primers of the primer pair contain sequence recognizable by one or more restriction endonucleases.

Following amplification, the relative enrichment of the target allele in the sample allows accurate detection of allele frequencies using practically any method of nucleic acid detection known in the art. For example, any of the following methods may be used, including, but not limited to, primer extension or microsequencing methods, ligase sequence determination methods, mismatch sequence determination methods, microarray sequence determination methods, restriction fragment length polymorphism (RFLP) procedures, PCR-based assays (e.g., TAQMAN® PCR System (Applied Biosystems)), nucleotide sequencing methods, hybridization methods, conventional dot blot analyses, single strand conformational polymorphism analysis (SSCP), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection, detection by mass spectrometry, real time-PCR and pyrosequencing.

The methods may also be multiplexed at high levels in a single reaction. For example, one or more alleles can be detected simultaneously. Multiplexing embodiments are particularly important when the genotype at a polymorphic locus is not known. In some instances, for example when the mother is heterozygous at the polymorphic locus, the assay may not be informative. See Figure 5A, which further describes the use of polymorphic variants to detect fetal nucleic acid from a maternal sample. In one embodiment, greater than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 300, 500 or more target alleles are assayed, wherein not every target allele is informative. In certain embodiments, the genotype at the polymorphic locus is known. In some embodiments, greater than 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 25, 30, 35, 40, 50 or more informative target alleles are assayed. The invention also in part includes combinations of different multiplex schemes provided herein.

In certain embodiments, the invention in part provides a method for quantifying a target allele at a polymorphic locus in a sample, wherein the sample contains nucleic acid, that comprises: digesting nucleic acid containing a maternal allele at the polymorphic locus with an enzyme, such as a restriction endonuclease, that selectively digests the maternal allele, wherein the selective digestion yields a DNA sample enriched for fetal DNA; determining the maternal or paternal allele frequency using polymorphic markers within the amplicon, and comparing the paternal or maternal allele frequency to a control DNA sample. In one embodiment, a difference in allele frequency is indicative of a chromosomal abnormality. In certain embodiments, the control DNA sample is a competitor oligonucleotide that is introduced to the assay in known quantities.

In certain embodiments, the present invention in part provides a kit for detecting the presence or absence of target nucleic acid. One component of the kit is primers for amplifying the region of interest. Another component of the kit comprises probes for discriminating between the different alleles of each nucleic acid species.

5

BRIEF DESCRIPTION OF THE FIGURES

Figure 1 is the HpyCH4V digest, which shows allele peak area ratios in a DNA mixture series. Peak area ratio is determined by dividing the calculated peak area of the SNP allele not recognized by HpyCH4V (*i.e.*, target allele) by the total peak area of both SNP alleles present in the mass spectrum.

10

Figure 2 is the NlaIII digest, which shows allele peak area ratios in a DNA mixture series. Peak area ratio is determined by dividing the calculated peak area of the SNP allele not recognized by NlaIII (*i.e.*, target allele) by the total peak area of both SNP alleles present in the mass spectrum.

15

Figure 3 is the HpyCH4V screenshots of 2% heterozygous DNA mixture. Note the appearance of the 'A' and 'T' alleles after HpyCH4V digestion of the DNA samples for rs4329520 and rs4658481, respectively.

Figure 4 is the NlaIII screenshots of 2% heterozygous DNA mixture. Note the appearance of the 'T' and 'A' alleles after NlaIII digestion of the DNA samples for rs2050927 and rs4329520, respectively.

20

Figure 5A shows the use of single nucleotide polymorphisms (SNP's) Fetal Identifiers to confirm the presence of fetal DNA by paternally-inherited alleles. Figure 5B shows representative mass spectra demonstrating the correlation between fetal DNA amounts estimated from AMG XY and from Fetal Identifier assays. The results were generated using the AMG primers provided in Figure 9A-9C.

25

Figure 6 depicts typical performance results for a qualified fetal identifier. Here the ability of the SNP assay to estimate the quantity of fetal DNA in the background of maternal DNA was verified for a total of 1700 copies and a total of 170 copies using genomic DNA mixtures. Note that the standard deviation of the estimate of fetal DNA increases due to the significant influence of the sampling error at low copy numbers.

30

Figure 7 shows the performance of multiplexed SNP assays (21 assays total) for detection of paternally-inherited alleles in a model system.

Figures 8A-8C provide the location design of the AMG primers. The amplification primers are underlined once and the extend primers are underlined twice. In addition, competitor sequences are provided. Competitor sequences may be used for quantitative methods. Figure 8C

35

includes a Results Table that shows the different masses generated by each of the AMG and SRY assays, which may be used to interpret the results from the assays.

Figure 9 provides the location design of the albumin (ALB) primers. The amplification primers are highlighted and the extend primer is underlined twice. Where the PCR primers are provided alone, the sequence-specific portion of the primer is underlined, and the multiplex tag is not underlined. In addition, competitor sequences are provided. Competitor sequences may be used for quantitative methods

DETAILED DESCRIPTION

It has been determined in the fields of biology and diagnostics that certain nucleic acids are present at very low concentrations in humans. In particular, fetal DNA has been found to exist in maternal plasma (Lo et al. *Lancet*. 1997 Aug 16;350(9076):485-7). This discovery has facilitated the development of non-invasive prenatal diagnostic approaches based simply on the analysis of a maternal blood sample (Lo et al. *Am J Hum Genet*. 1998 Apr;62(4):768-75). The non-invasive nature of maternal plasma-based approaches represents a major advantage over conventional methods of prenatal diagnosis, such as amniocentesis and chorionic villus sampling, which are associated with a small but finite risk of fetal loss. However, a technical challenge experienced by many workers in the field relates to the ability to discriminate the relatively small amount of fetal DNA from the coexisting background of maternal DNA in maternal plasma. During pregnancy, fetal DNA amounts to approximately 3–6% of the total DNA in maternal plasma. Hence, the diagnostic reliability of fetal DNA analysis in maternal plasma generally has depended on the accurate detection of fetal-specific markers.

Methods described herein solve this problem by enriching, relatively, the amount of low copy number nucleic acid before detecting or quantifying the alleles present in the sample. In the case of prenatal diagnostics, the use of restriction endonuclease enhanced polymorphic sequence detection allows for the selective, sensitive detection of fetal nucleic acid from maternal samples. The fetal DNA in the maternal plasma sample is selectively enriched before detecting the alleles present in the maternal sample. To enrich for fetal DNA present in plasma of the mother to allow accurate detection of fetal alleles present in the sample, the methods described herein allow for the cleavage of maternal nucleic acid or nucleic acid of maternal origin. Thus, the maternal DNA can be substantially reduced, masked, or destroyed completely, and the sample is left with DNA enriched for DNA of fetal origin. The selective reduction of maternal DNA can be performed using one or more enzymes, such as restriction endonucleases, which selectively digest nucleic acids which comprise maternal alleles.

Definitions

The term “sample” as used herein includes a specimen or culture (e.g., microbiological cultures) that includes nucleic acids. The term “sample” is also meant to include both biological and environmental samples. A sample may include a specimen of synthetic origin. Biological samples include whole blood, serum, plasma, umbilical cord blood, chorionic villi, amniotic fluid, cerebrospinal fluid, spinal fluid, lavage fluid (e.g., bronchoalveolar, gastric, peritoneal, ductal, ear, athroscopic), biopsy sample, urine, feces, sputum, saliva, nasal mucous, prostate fluid, semen, lymphatic fluid, bile, tears, sweat, breast milk, breast fluid, embryonic cells and fetal cells. The biological sample can be maternal blood, including maternal plasma or serum. In some circumstances, the biological sample is acellular. In other circumstances, the biological sample does contain cellular elements or cellular remnants in maternal blood.

In a preferred embodiment, the sample comprises a mixture of nucleic acids. For example, the mixture may comprise nucleic acid from different species or from different individuals. In one embodiment, the sample is from a pregnant female or a female suspected of being pregnant. In a related embodiment, the sample is procured through non-invasive means (e.g., a blood draw). In certain embodiments the sample is from any animal, including but not limited, human, non-human, mammal, reptile, cattle, cat, dog, goat, swine, pig, monkey, ape, gorilla, bull, cow, bear, horse, sheep, poultry, mouse, rat, fish, dolphin, whale, and shark, or any animal or organism that may be tested for the presence of target nucleic acid.

In a preferred embodiment, the biological sample is blood, and more preferably plasma. As used herein, the term “blood” encompasses whole blood or any fractions of blood, such as serum and plasma as conventionally defined. Blood plasma refers to the fraction of whole blood resulting from centrifugation of blood treated with anticoagulants. Blood serum refers to the watery portion of fluid remaining after a blood sample has coagulated. Environmental samples include environmental material such as surface matter, soil, water, crime scene samples, and industrial samples, as well as samples obtained from food and dairy processing instruments, apparatus, equipment, utensils, disposable and non-disposable items. These examples are not to be construed as limiting the sample types applicable to the present invention.

The term “non-invasive” as used herein refers a method for collecting a sample that poses minimal risk to an individual (e.g., the mother, fetus, victim, etc.). An example of a non-invasive method is a blood draw; whereas examples of invasive methods include amniocentesis and chorionic villus sampling, both of which constitute a finite risk to the fetus.

The terms “target” or “target nucleic acid” as used herein are intended to mean any molecule whose presence is to be detected or measured or whose function, interactions or properties are to be studied, wherein target nucleic acid comprises the target allele and non-target

nucleic acid comprises the non-target allele. Fetal nucleic acid may comprise both target nucleic acid and non-target nucleic when the fetus is heterozygous at a polymorphic locus. Other examples of target nucleic acid include, but are not limited to, trace nucleic acid, mutated nucleic acid, viral nucleic acid and transplant nucleic acid.

5 The terms "nucleic acid" and "nucleic acid molecule" may be used interchangeably throughout the disclosure. The terms refer to oligonucleotides, oligos, polynucleotides, deoxyribonucleotide (DNA), genomic DNA, mitochondrial DNA (mtDNA), complementary DNA (cDNA), bacterial DNA, viral DNA, viral RNA, RNA, message RNA (mRNA), transfer RNA (tRNA), ribosomal RNA (rRNA), siRNA, catalytic RNA, clones, plasmids, M13, P1, cosmid, bacteria artificial
10 chromosome (BAC), yeast artificial chromosome (YAC), amplified nucleic acid, amplicon, PCR product and other types of amplified nucleic acid, RNA/DNA hybrids and polyamide nucleic acids (PNAs), all of which can be in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally occurring nucleotides and combinations and/or mixtures thereof. Thus, the term
15 "nucleotides" refers to both naturally-occurring and modified/non-naturally-occurring nucleotides, including nucleoside tri, di, and monophosphates as well as monophosphate monomers present within polynucleic acid or oligonucleotide. A nucleotide may also be a ribo; 2'-deoxy; 2', 3'-deoxy as well as a vast array of other nucleotide mimics that are well-known in the art. Mimics include chain-terminating nucleotides, such as 3'-O-methyl, halogenated base or sugar substitutions;
20 alternative sugar structures including nonsugar, alkyl ring structures; alternative bases including inosine; deaza-modified; chi, and psi, linker-modified; mass label-modified; phosphodiester modifications or replacements including phosphorothioate, methylphosphonate, boranophosphate, amide, ester, ether; and a basic or complete internucleotide replacements, including cleavage linkages such as a photocleavable nitrophenyl moieties.

25 In the case of RNA, the RNA may be placentally-expressed RNA in maternal plasma. Background maternal RNA may be selectively digested according to the methods provided herein. Also, the method may further comprise the additional step of discriminating the alleles of RNA which involves reverse transcriptase polymerase chain reaction (RT-PCR). In certain embodiments, the fetal RNA may be extracted from maternal body fluids, preferably whole blood,
30 and more preferably plasma or serum using e. g. RNA extraction methods such as, but not limited to, gelatin extraction method; silica, glass bead, or diatom extraction method; guanidinium thiocyanate acid- phenol based extraction methods; guanidinium thiocyanate acid based extraction methods; guanidine-hydrochloride based extraction methods; methods using centrifugation through cesium chloride or similar gradients; phenol-chloroform based extraction methods; and/or other
35 available RNA extraction methods, as are known in the art for use in extraction of intracellular

RNA, including commercially available RNA extraction methods, e. g. by using or adapting or modifying the methods of Boom et al. (1990, J. Clin. Microbiol. 28: 495-503); Cheung et al. (1994, J. Clin. Microbiol. 32: 2593-2597); Boom et al. (1991, J. Clin. Microbiol. 29: 1804-1811); Chomczynski and Sacchi (1987, Analytical Biochem. 162: 156-159); Chomczynski, (1993, Biotech. 15: 532-537); Chomczynski and Mackey (1995, Biotechniques 19: 942-945); Chomczynski and Mackey (1995, Anal. Biochem. 225: 163-164); Chirgwin et al. (1979, Biochem. 18: 5294-5299); Fournie et al. (1986 Anal. Biochem. 158: 250-256); and W097/35589.

The term “amplification reaction” refers to any in vitro means for multiplying the copies of nucleic acid.

“Amplifying” refers to a step of submitting a sample to conditions sufficient to allow for amplification. Components of an amplification reaction may include, but are not limited to, e.g., primers, a polynucleotide template, polymerase, nucleotides, dNTPs and the like. The term “amplifying” typically refers to an “exponential” increase in target nucleic acid. However, “amplifying” as used herein can also refer to linear increases in the numbers of a select target sequence of nucleic acid, but is different than a one-time, single primer extension step.

“Polymerase chain reaction” or “PCR” refers to a method whereby a specific segment or subsequence of a target double-stranded DNA, is amplified in a geometric progression. PCR is well known to those of skill in the art; see, e.g., U.S. Pat. Nos. 4,683,195 and 4,683,202; and PCR Protocols: A Guide to Methods and Applications, Innis et al., eds, 1990.

“Oligonucleotide” as used herein refers to linear oligomers of natural or modified nucleosidic monomers linked by phosphodiester bonds or analogs thereof. Oligonucleotides include deoxyribonucleosides, ribonucleosides, anomeric forms thereof, peptide nucleic acids (PNAs), and the like, capable of specifically binding to a target nucleic acid. Usually monomers are linked by phosphodiester bonds or analogs thereof to form oligonucleotides ranging in size from a few monomeric units, e.g., 3-4, to several tens of monomeric units, e.g., 40-60. Whenever an oligonucleotide is represented by a sequence of letters, such as “ATGCCTG,” it will be understood that the nucleotides are in 5'-3' order from left to right and that “A” denotes deoxyadenosine, “C” denotes deoxycytidine, “G” denotes deoxyguanosine, “T” denotes deoxythymidine, and “U” denotes the ribonucleoside, uridine, unless otherwise noted. Oligonucleotides often comprise the four natural deoxynucleotides; however, they may also comprise ribonucleosides or non-natural nucleotide analogs. Where an enzyme has specific oligonucleotide or polynucleotide substrate requirements for activity, e.g., single stranded DNA, RNA/DNA duplex, or the like, then selection of appropriate composition for the oligonucleotide or polynucleotide substrates is well within the knowledge of one of ordinary skill.

As used herein "oligonucleotide primer", or simply "primer", refers to a polynucleotide sequence that hybridizes to a sequence on a nucleic acid template and facilitates the amplification of the nucleic acid template, or otherwise plays a role in the detection of the nucleic acid molecule. In amplification embodiments, an oligonucleotide primer serves as a point of initiation of nucleic acid synthesis. Primers can be of a variety of lengths and are often less than 50 nucleotides in length, for example 12-25 nucleotides, in length. The length and sequences of primers for use in PCR can be designed based on principles known to those of skill in the art.

The term "template" refers to any nucleic acid molecule that can be used for amplification in the methods described herein. RNA or DNA that is not naturally double stranded can be made into double stranded DNA so as to be used as template DNA. Any double stranded DNA or preparation containing multiple, different double stranded DNA molecules can be used as template DNA to amplify a locus or loci of interest contained in the template DNA.

The term "amplicon" as used herein refers to amplified DNA that has been "copied" once or multiple times, e.g. by polymerase chain reaction. The amplicon sequence falls between the amplification primers.

The term "polymorphic locus" as used herein refers to a nucleic acid region that comprises a polymorphism. The nucleic acid region may be 1, 2, 3, 4, 5, 6, 7, 8, 9, 10, 11, 12, 13, 14, 15, 20, 25, 30, 35, 40, 50, 60, 70, 80, 90, 100, 200, 300, 400, 500 or more nucleotides in length.

The term "polymorphism" as used herein refers to an allelic variant. Polymorphisms can include single nucleotide polymorphisms (SNP's) as well as simple sequence length polymorphisms. A polymorphism can be due to one or more nucleotide substitutions at one allele in comparison to another allele or can be due to an insertion or deletion, duplication, inversion and other alterations known to the art. Other polymorphisms include, but are not limited to, restriction fragment length polymorphisms (RFLPs), insertions/deletions, short tandem repeats, such as di-, tri- or tetra-nucleotide repeats (STRs), and the like. As used herein, polymorphism may include epigenetic variants, as long as cleavage by non-epigenetic-specific cleavage agents is used.

The term "allele" as used herein is one of several alternate forms of a gene or non-coding regions of DNA that occupy the same position on a chromosome. The term allele can be used to describe DNA from any organism including but not limited to bacteria, viruses, fungi, protozoa, molds, yeasts, plants, humans, non-humans, animals, and archeabacteria.

Alleles can have the identical sequence or can vary by a single nucleotide or more than one nucleotide. With regard to organisms that have two copies of each chromosome, if both chromosomes have the same allele, the condition is referred to as homozygous. If the alleles at the two chromosomes are different, the condition is referred to as heterozygous. For example, if the locus of interest is SNP X on chromosome 1, and the maternal chromosome contains an adenine

at SNP X (A allele) and the paternal chromosome contains a guanine at SNP X (G allele), the individual is heterozygous A/G at SNP X.

As used herein, the term "mutant alleles" may refer to variant alleles that are associated with a disease state, e.g., cancer.

5 The term "sequence identifier" as used herein refers to any sequence difference that exists between two sequences that can be used to differentiate the sequences. In one embodiment, the sequence identifier does not include methylation differences.

As used herein, the term "genotype" refers to the identity of the alleles or non-homologous variants present in an individual or sample. The term "genotyping a sample" or "genotyping an individual" refers to determining a specific allele or specific nucleotide(s) or polymorphism(s) in a sample or carried by an individual at particular region(s).

The term "selectively" as used herein is not intended to suggest an absolute event, but instead a preferential event. For example, "selectively cleaved" is used to indicate one sequence (for example, the non-target sequence) is preferentially cleaved or digested over another sequence (for example, the target sequence). However, some of the target sequence may also be cleaved due to a lack of specificity with the cleavage agent or other variables introduced during the cleavage process.

The term "cleavage agent" as used herein refers to any means that is capable of differentially cleaving two or more sequences based on a sequence difference that exists between the two or more sequences. The cleavage agent may be an enzyme. The cleavage agent may be natural, synthetic, unmodified or modified. In a preferred embodiment, the cleavage agent is a restriction endonuclease. Restriction endonucleases, alternatively called restriction enzymes, are a class of bacterial enzymes that cut or digest DNA at specific sites. Type I restriction endonucleases occur as a complex with the methylase and a polypeptide that binds to the recognition site on DNA. They are often not very specific and cut at a remote site. Type II restriction endonucleases are the classic experimental tools. They have very specific recognition and cutting sites. The recognition sites are short, 4-8 nucleotides, and are usually palindromic sequences. Because both strands have the same sequence running in opposite directions the enzymes make double-stranded breaks, which, if the site of cleavage is off-center, generates fragments with short single-stranded tails; these can hybridize to the tails of other fragments and are called sticky ends. They are generally named according to the bacterium from which they were isolated (first letter of genus name and the first two letters of the specific name). The bacterial strain is identified next and multiple enzymes are given Roman numerals. For example the two enzymes isolated from the R strain of E. coli are designated Eco RI and Eco RII. In a preferred

embodiment, the restriction enzyme is a type II restriction endonuclease. In another preferred embodiment, the restriction enzyme is non-thermostable.

The term "chromosomal abnormality" as used herein refers to a deviation between the structure of the subject chromosome and a normal homologous chromosome. The term "normal" refers to the predominate karyotype or banding pattern found in healthy individuals of a particular species. A chromosomal abnormality can be numerical or structural, and includes but is not limited to aneuploidy, polyploidy, inversion, a trisomy, a monosomy, duplication, deletion, deletion of a part of a chromosome, addition, addition of a part of chromosome, insertion, a fragment of a chromosome, a region of a chromosome, chromosomal rearrangement, and translocation. A chromosomal abnormality can be correlated with presence of a pathological condition or with a predisposition to develop a pathological condition.

Uses and advantages associated with the methods described herein

The invention in part provides nucleic acid-based assays that are particularly useful for non-invasive prenatal testing. The methods provided herein may be used, *inter alia*, to determine the presence of fetal nucleic acid in a sample, to determine the amount of fetal nucleic acid in a sample, to determine the sex of a fetus, and to enrich for a target nucleic acid sequence. The invention in part may be combined with other prenatal methods, such as those described in US Application No. 12/027,954, filed February 7, 2008,; PCT Application No. PCT/US07/69991, filed May 30, 2007; PCT Application No. PCT/US07/071232, filed June 15, 2007; US Provisional Application No. 61/033,343, filed March 3, 2008; US Provisional Application No. 61/035,711, filed March 11, 2008; or any of the prenatal diagnostic (both invasive and non-invasive) methods disclosed in US Provisional Application No. 60/944,331, filed June 15, 2007, all of which are hereby incorporated by reference.

The invention in part may be used to more accurately detect fetal DNA using high frequency polymorphisms that match the criteria provided herein. These polymorphisms are alternatively called fetal identifiers. The criteria includes one or more of the following:

- 1) One allele of the SNP is recognized by the cleavage agent;
- 2) The alternate SNP allele is not recognized by the same cleavage agent;
- 3) No other sites for the cleavage are found +/- 50 base pair of the SNP within the PCR amplicon; and
- 4) (Optionally) The minor allele frequency is greater than 0.4 (preferably across a range of populations).

Examples of fetal identifiers are set forth in Tables 6, 9, 10 and 11. In one embodiment, the method of detecting the presence or absence of fetal nucleic acid in a sample comprises obtaining

or possessing a nucleic acid sample known to be of maternal origin and suspected of comprising fetal nucleic acid; analyzing the nucleic acid sample to determine the maternal genotype at one or more nucleotide polymorphisms selected from the group consisting of the polymorphisms set forth in Tables 6, 9, 10 and 11; and analyzing the nucleic acid sample to determine the fetal genotype of one or more nucleotide polymorphisms selected from the group consisting of the polymorphisms set forth in Tables 6, 9, 10 and 11, wherein a fetal genotype possessing a paternally-inherited allele indicates the presence of fetal nucleic acid, further wherein nucleic acid comprising a maternal allele is digested using the methods provided herein. In a preferred embodiment, one or more of the polymorphisms set forth in Table 6 or 11 are used in conjunction with the methods provided herein. In a related embodiment, the maternal genotypes are first determined from DNA that is substantially free of fetal nucleic acid. For example, in the case when the sample is blood, the maternal genotypes may be determined from the portion of the blood that comprises nucleated maternal cells (e.g., white blood cells). In one embodiment, the DNA that is substantially free of fetal nucleic acid is from peripheral blood mononuclear cells. In certain embodiments, the amount of fetal DNA is determined by comparing the relative amount of paternally-inherited alleles to an internal control (e.g., competitor oligonucleotide).

In Tables 6, 9, 10 and 11, each primer of the amplification primer pair may comprise the entire sequence shown or only the non-underlined sequence, wherein the underlined portion of the primer is a tag sequence (ACGTTGGATG) for improved multiplexing and the non-underlined portion is a sequence-specific primer sequence. The tag sequence may be any tag sequence known in the art that improves multiplexing. In certain embodiments, the invention in part includes primers that are substantially similar to the primers provided herein, for example, about 90% or more similar, and further wherein the primers are still specific for a given nucleic acid region. For example, one or more bases of a primer sequence may be changed or substituted, for example with an inosine, but the primer still maintains the same specificity and plexing ability. Bases indicated by uppercase text are complementary to the nucleic acid sequence to which the primer hybridizes, and bases indicated by lowercase text are not complementary to the nucleic acid sequence to which the primer hybridizes. Bases indicated in lower case text can be selected to shift or adjust the mass of primers and amplification products.

In particular embodiments, a sequence tag is attached to a plurality of primary and secondary primer pairs provided in Tables 6, 9, 10 and 11. The sequence tag can be attached to either one or both of the primary and secondary primers from each pair. Typically, the sequence tag is attached to the primary and secondary primer of each pair. The sequence tags used herein can range from 5 up to 20, from 5 up to 30, from 5 up to 40, or from 5 up to 50 nucleotides in length, with a sequence tag of 10-mer length being particularly useful in the methods provided

herein. The sequence tag need not be the same sequence for each primer pair in the multiplexed amplification reaction, nor the same sequence for a primary and secondary primer within a particular amplification pair. In a particular embodiment, the sequence tag is the same for each primer in the multiplexed amplification reaction. For example, in certain embodiments, the
5 sequence tag is a 10-mer, such as -ACGTTGGATG-, and is attached to the 5' end of each primary and secondary primer. In particular embodiments of the methods provided herein, only a single primer pair is used to amplify each particular nucleic acid target-region.

In certain embodiments, methods described herein may be used to improve the detection the Y-chromosome in a maternal sample, which may be used to determine the sex of a fetus. The
10 presence or absence of the Y-chromosome in a maternal sample may be determined by performing the SRY assay described in Example 3. The SRY assay is a highly sensitive quantitative internal standard assay that detects trace amounts of the Y-chromosome. In certain embodiments, other polymorphisms located on the Y-chromosome may be assayed according to the methods provided herein.

15 The presence or absence of the Y-chromosome in a maternal sample may also be determined by performing the AMG assay provided herein. The presence or absence of a target nucleic acid may be determined in combination with other assays, such as an RhD assay, blood type assay or sex test assay. The methods may also be used for other applications, including but not limited to, paternity testing, forensics or quality control assays.

20 In addition to prenatal applications, the methods find utility in a range of applications, including, but not limited to, detecting rare cancer mutations, detecting transplant rejection and forensics.

In certain embodiments, the total copy number of nucleic acid molecules for the human serum albumin (ALB) gene is determined. Methods for determining the total copy number of
25 nucleic acid present in a sample comprise detecting albumin-specific extension products and comparing the relative amount of the extension products to competitors introduced to the sample. In a related embodiment, the invention in part provides compositions and methods to determine the relative amount of fetal DNA in a sample (e.g., when the sample is plasma from a pregnant woman carrying a male fetus), which comprises annealing one or more albumin gene sequences to the
30 fetal DNA, the primers provided in Figure 9; performing a primer extension reaction; and analyzing the primer extension products to determine the relative amount of ALB extension products, wherein maternal albumin nucleic acid has been reduced using the methods provided herein. In a related embodiment, the fetal ALB amplicon is first amplified using the amplification primers provided in Figure 9. The assay is useful to measure how much nucleic acid (e.g., total copy number) is
35 present in a sample or loaded into a particular reaction. The assay may serve as an internal

control and a guide to the likelihood of success for a particular PCR reaction. For example, if only 400 copies of ALB are measured then the probability of detecting any fetal DNA may be considered low. In certain embodiments, the competitors provided in Figure 9 are introduced as an internal standard to determine copy number. In one embodiment, 200, 300, 400, 500, 600, 700, 800 or more competitor molecules are introduced to the assay.

Methods described herein provide a number of advantageous. The methods allow a high sensitivity to detect polymorphic alleles (e.g., fetal identifiers) present at low relative percentages in a DNA mixture and present at low copy number. The methods may also be incorporated into multiplexed assays in a single reaction. The methods are readily implemented, and only add a single additional step to the many current detection methods.

Nucleases

Cleavage methods and procedures for selecting restriction enzymes for cutting nucleic acid at specific sites are well known to the skilled artisan. For example, many suppliers of restriction enzymes provide information on conditions and types of DNA sequences cut by specific restriction enzymes, including New England BioLabs, Pro-Mega Biochems, Boehringer-Mannheim, and the like.

The preparation of nucleic acid to be cleaved should be free of contaminants such as phenol, chloroform, alcohol, EDTA, detergents, or excessive salts, all of which can interfere with restriction enzyme activity.

Embodiments of the invention can be assembled from multiple restriction endonucleases (available from various vendors) that are chosen to correspond to appropriate polymorphic alleles, as long as the restriction endonuclease selects for one polymorphic allele over another and performs a digestion within the amplicon sequence such that it prevents a subsequent amplification event. In one embodiment, the amplicon is chosen such that it contains a variable nuclease restriction site and sequence identifier, which may or may not be the same as the restriction site. Also, the restriction enzyme need not cleave at the polymorphic site, for example, at the variable nucleotide of a SNP.

Restriction enzymes are traditionally classified into three types on the basis of subunit composition, cleavage position, sequence-specificity and cofactor-requirements. However, amino acid sequencing has uncovered extraordinary variety among restriction enzymes and revealed that at the molecular level there are many more than three different kinds.

Type I enzymes are complex, multisubunit, combination restriction-and-modification enzymes that cut DNA at random far from their recognition sequences. Originally thought to be rare, we now know from the analysis of sequenced genomes that they are common. Type I

enzymes are of considerable biochemical interest but they have little practical value since they do not produce discrete restriction fragments or distinct gel-banding patterns.

Type II enzymes cut DNA at defined positions close to or within their recognition sequences. They produce discrete restriction fragments and distinct gel banding patterns, and they are the only class used in the laboratory for DNA analysis and gene cloning. Type II enzymes frequently differ so utterly in amino acid sequence from one another, and indeed from every other known protein, that they likely arose independently in the course of evolution rather than diverging from common ancestors.

The most common type II enzymes are those like HhaI, HindIII and NotI that cleave DNA within their recognition sequences. Enzymes of this kind are the principle ones available commercially. Most recognize DNA sequences that are symmetric because they bind to DNA as homodimers, but a few, (e.g., BbvCI: CCTCAGC) recognize asymmetric DNA sequences because they bind as heterodimers. Some enzymes recognize continuous sequences (e.g., EcoRI: GAATTC) in which the two half-sites of the recognition sequence are adjacent, while others recognize discontinuous sequences (e.g., BglI: GCCNNNNNGGC) in which the half-sites are separated. Cleavage leaves a 3'-hydroxyl on one side of each cut and a 5'-phosphate on the other. They require only magnesium for activity and the corresponding modification enzymes require only S-adenosylmethionine. They tend to be small, with subunits in the 200–350 amino acid range.

The next most common type II enzymes, usually referred to as 'type IIs' are those like FokI and AlwI that cleave outside of their recognition sequence to one side. These enzymes are intermediate in size, 400–650 amino acids in length, and they recognize sequences that are continuous and asymmetric. They comprise two distinct domains, one for DNA binding, the other for DNA cleavage. They are thought to bind to DNA as monomers for the most part, but to cleave DNA cooperatively, through dimerization of the cleavage domains of adjacent enzyme molecules. For this reason, some type IIs enzymes are much more active on DNA molecules that contain multiple recognition sites. A wide variety of Type IIS restriction enzymes are known and such enzymes have been isolated from bacteria, phage, archeabacteria and viruses of eukaryotic algae and are commercially available (Promega, Madison Wis.; New England Biolabs, Beverly, Mass.). Examples of Type IIS restriction enzymes that may be used with the methods described herein include, but are not limited to enzymes such as those listed in Table IA.

WO 2008/118988

PCT/US2008/058317

TABLE 1A

Enzyme-Source	Recognition/Cleavage Site	Supplier
Alw I - <i>Acinetobacter lwoffii</i>	GGATC(4/5)	NE Biolabs
Alw26 I - <i>Acinetobacter lwoffii</i>	GTCTC(1/5)	Promega
Bbs I - <i>Bacillus laterosporus</i>	GAAGAC(2/6)	NE Biolabs
Bbv I - <i>Bacillus brevis</i>	GCAGC(8/12)	NE Biolabs
BceA I - <i>Bacillus cereus</i> 1315	IACGGC(12/14)	NE Biolabs
Bmr I - <i>Bacillus megaterium</i>	CTGGG(5/4)	NE Biolabs
Bsa I - <i>Bacillus stearothermophilus</i> 6-55	GGTCTC(1/5)	NE Biolabs
Bst71 I - <i>Bacillus stearothermophilus</i> 71	GCAGC(8/12)	Promega
BsmA I - <i>Bacillus stearothermophilus</i> A664	GTCTC(1/5)	NE Biolabs
BsmB I - <i>Bacillus stearothermophilus</i> B61	CGTCTC(1/5)	NE Biolabs
BsmF I - <i>Bacillus stearothermophilus</i> F	GGGAC(10/14)	NE Biolabs
BspM I - <i>Bacillus species</i> M	ACCTGC(4/8)	NE Biolabs
Ear I - <i>Enterobacter aerogenes</i>	CTCTTC(1/4)	NE Biolabs
Fau I - <i>Flavobacterium aquatile</i>	CCCGC(4/6)	NE Biolabs
Fok I - <i>Flavobacterium okeonokoites</i>	GGATG(9/13)	NE Biolabs
Hga I - <i>Haemophilus gallinarum</i>	GACGC(5/10)	NE Biolabs
Ple I - <i>Pseudomonas lemoignei</i>	GAGTC(4/5)	NE Biolabs
Sap I - <i>Saccharopolyspora species</i>	GCTCTTC(1/4)	NE Biolabs
SfaN I - <i>Streptococcus faecalis</i> ND547	GCATC(5/9)	NE Biolabs
Sth132 I - <i>Streptococcus thermophilus</i> ST132	CCCG(4/8)	No commercial supplier (Gene 195: 201-206 (1997))

The third major kind of type II enzyme, more properly referred to as “type IV” are large, combination restriction-and-modification enzymes, 850–1250 amino acids in length, in which the two enzymatic activities reside in the same protein chain. These enzymes cleave outside of their recognition sequences; those that recognize continuous sequences (e.g., Eco57I: CTGAAG) cleave on just one side; those that recognize discontinuous sequences (e.g., BcgI: CGANNNNNNTGC) cleave on both sides releasing a small fragment containing the recognition sequence. The amino acid sequences of these enzymes are varied but their organization are consistent. They comprise an N-terminal DNA-cleavage domain joined to a DNA-modification domain and one or two DNA sequence-specificity domains forming the C-terminus, or present as a separate subunit. When these enzymes bind to their substrates, they switch into either restriction mode to cleave the DNA, or modification mode to methylate it.

As discussed above, the length of restriction recognition sites varies. For example, the enzymes EcoRI, SacI and SstI each recognize a 6 base-pair (bp) sequence of DNA, whereas NotI recognizes a sequence 8 bp in length, and the recognition site for Sau3AI is only 4 bp in length. Length of the recognition sequence dictates how frequently the enzyme will cut in a random sequence of DNA. Enzymes with a 6 bp recognition site will cut, on average, every 4⁶ or 4096 bp; a 4 bp recognition site will occur roughly every 256 bp.

Different restriction enzymes can have the same recognition site - such enzymes are called isoschizomers. Table IB shows that the recognition sites for SacI and SstI are identical. In some cases isoschizomers cut identically within their recognition site, but sometimes they do not. Isoschizomers often have different optimum reaction conditions, stabilities and costs, which may influence the decision of which to use. Table IB is provided only to show exemplary restriction enzymes, and does not limit the scope of the invention in any way.

TABLE IB

Enzyme	Recognition Sequence
BamH I	GGATCC CCTAGG
Not I	GCGGCCGC CGCCGGCG
Sau3A I	GATC CTAG
Sac I	GAGCTC CTCGAG
Sst I	GAGCTC CTCGAG
Hinf I	GANTC CTNAG
Xho II	PuGATCPy PyCTAGPu

Restriction recognitions sites can be unambiguous or ambiguous. The enzyme BamHI recognizes the sequence GGATCC and no others; therefore it is considered “unambiguous.” In contrast, HinfI recognizes a 5 bp sequence starting with GA, ending in TC, and having any base between (in Table IB, “N” stands for any nucleotide). HinfI has an ambiguous recognition site. XhoII also has an ambiguous recognition site: Py stands for pyrimidine (T or C) and Pu for purine (A or G), so XhoII will recognize and cut sequences of AGATCT, AGATCC, GGATCT and GGATCC.

The recognition site for one enzyme may contain the restriction site for another. For example, note that a BamHI recognition site contains the recognition site for Sau3AI.

Consequently, all BamHI sites will cut with Sau3AI. Similarly, one of the four possible XhoII sites will also be a recognition site for BamHI and all four will cut with Sau3AI.

Also from Table IB, most recognition sequences are palindromes - they read the same forward (5' to 3' on the top strand) and backward (5' to 3' on the bottom strand). Most, but certainly not all recognition sites for commonly-used restriction enzymes are palindromes. Most restriction enzymes bind to their recognition site as dimers (pairs).

Nucleic acid detection

Whether detecting sequence differences, detecting amplification products or primer extension products, any detection or discrimination method known in the art may be utilized. These methods include, but are not limited to, primer extension reactions, mass spectrometry, hybridization using at least one probe, hybridization using at least one fluorescently labeled probe, direct sequencing, cloning and sequencing, and electrophoresis. Polymorphism detection methods known in the art may also include, for example, microsequencing methods, ligase sequence determination methods (e.g., U.S. Pat. Nos. 5,679,524 and 5,952,174, and WO 01/27326), digital PCR (U.S. Pat. No. 6,143,496), mismatch sequence determination methods (e.g., U.S. Pat. Nos. 5,851,770; 5,958,692; 6,110,684; and 6,183,958), microarray sequence determination methods, restriction fragment length polymorphism (RFLP) procedures, PCR-based assays (e.g., TAQMAN[®] PCR System (Applied Biosystems)), nucleotide sequencing methods, hybridization methods, conventional dot blot analyses, single strand conformational polymorphism analysis (SSCP, e.g., U.S. Patent Nos. 5,891,625 and 6,013,499; Orita *et al.*, *Proc. Natl. Acad. Sci. U.S.A* 86: 27776-2770 (1989)), denaturing gradient gel electrophoresis (DGGE), heteroduplex analysis, mismatch cleavage detection, and techniques described in Sheffield *et al.*, *Proc. Natl. Acad. Sci. USA* 49: 699-706 (1991), White *et al.*, *Genomics* 12: 301-306 (1992), Grompe *et al.*, *Proc. Natl. Acad. Sci. USA* 86: 5855-5892 (1989), and Grompe, *Nature Genetics* 5: 111-117 (1993), detection by mass spectrometry (e.g., US 20050079521, which is hereby incorporated by reference), real time-PCR (e.g., US Patent Nos. US 5,210,015, US 5,487,972, both of which are hereby incorporated by reference), or hybridization with a suitable nucleic acid primer specific for the sequence to be detected. Suitable nucleic acid primers can be provided in a format such as a gene chip.

Primer extension polymorphism detection methods, also referred to herein as "microsequencing" methods, typically are carried out by hybridizing a complementary oligonucleotide to a nucleic acid carrying the polymorphic site. In these methods, the oligonucleotide typically hybridizes adjacent to the polymorphic site. As used herein, the term "adjacent" refers to the 3' end of the extension oligonucleotide being sometimes 1 nucleotide from the 5' end of the polymorphic site, often 2 or 3, and at times 4, 5, 6, 7, 8, 9, or 10 nucleotides from

the 5' end of the polymorphic site, in the nucleic acid when the extension oligonucleotide is hybridized to the nucleic acid. The extension oligonucleotide then is extended by one or more nucleotides, often 1, 2, or 3 nucleotides, and the number and/or type of nucleotides that are added to the extension oligonucleotide determine which polymorphic variant or variants are present.

5 Oligonucleotide extension methods are disclosed, for example, in U.S. Patent Nos. 4,656,127; 4,851,331; 5,679,524; 5,834,189; 5,876,934; 5,908,755; 5,912,118; 5,976,802; 5,981,186; 6,004,744; 6,013,431; 6,017,702; 6,046,005; 6,087,095; 6,210,891; and WO 01/20039. The extension products can be detected in any manner, such as by fluorescence methods (see, e.g., Chen & Kwok, *Nucleic Acids Research* 25: 347-353 (1997) and Chen *et al.*, *Proc. Natl. Acad. Sci.* 10 *USA* 94/20: 10756-10761 (1997)) and by mass spectrometric methods (e.g., MALDI-TOF mass spectrometry). Oligonucleotide extension methods using mass spectrometry are described, for example, in U.S. Patent Nos. 5,547,835; 5,605,798; 5,691,141; 5,849,542; 5,869,242; 5,928,906; 6,043,031; 6,194,144; and 6,258,538.

Microsequencing detection methods often incorporate an amplification process that 15 proceeds the extension step. The amplification process typically amplifies a region from a nucleic acid sample that comprises the polymorphic site. Amplification can be carried out by utilizing a pair of oligonucleotide primers in a polymerase chain reaction (PCR), in which one oligonucleotide primer typically is complementary to a region 3' of the polymorphism and the other typically is complementary to a region 5' of the polymorphism. A PCR primer pair may be used in methods 20 disclosed in U.S. Patent Nos. 4,683,195; 4,683,202; 4,965,188; 5,656,493; 5,998,143; 6,140,054; WO 01/27327; and WO 01/27329 for example. PCR primer pairs may also be used in any commercially available machines that perform PCR, such as any of the GENEAMP® Systems available from Applied Biosystems.

A microarray can be utilized for determining whether a polymorphic variant is present or 25 absent in a nucleic acid sample. A microarray may include any oligonucleotides described herein, and methods for making and using oligonucleotide microarrays suitable for prognostic use are disclosed in U.S. Pat. Nos. 5,492,806; 5,525,464; 5,589,330; 5,695,940; 5,849,483; 6,018,041; 6,045,996; 6,136,541; 6,142,681; 6,156,501; 6,197,506; 6,223,127; 6,225,625; 6,229,911; 6,239,273; WO 00/52625; WO 01/25485; and WO 01/29259. The microarray typically comprises a 30 solid support and the oligonucleotides may be linked to this solid support by covalent bonds or by non-covalent interactions. The oligonucleotides may also be linked to the solid support directly or by a spacer molecule. A microarray may comprise one or more oligonucleotides complementary to a polymorphic site within a nucleotide sequence.

EXAMPLES

The following examples are provided to further describe embodiments of the invention and are not limiting.

5

Example 1: Restriction Endonuclease Enhanced Polymorphic Sequence Detection Using Hpych4v And NlaIII

The effectiveness of restriction endonuclease enhanced polymorphic sequence detection was demonstrated using several restriction endonucleases (REs), including HpyCH4V and NlaIII (purchased from New England BioLabs, Inc). Both of these enzymes were separately tested in multiplexed genotyping reactions for their ability to specifically cleave one allele of a given polymorphism while allowing PCR amplification of the remaining allele of the polymorphism. See Table 2 for the polymorphisms tested with each enzyme.

Two CEPH DNA samples were mixed in varying ratios to generate DNA samples composed of 0%, 2%, 5%, 20%, 50% and 100% DNA heterozygous for both alleles of the SNP, with the remaining DNA being homozygous for the allele recognized by the RE. Table 3 shows DNA samples used in these studies and corresponding genotype information. Mixtures composed of NA05995 and NA10849 were used for experiments with HpyCH4V enzyme, and mixtures composed of NA10862 and NA10846 were used for experiments with NlaIII enzyme.

TABLE 2: Restriction enzymes recognizing SNPs

Restriction Enzyme	Polymorphism	SNP Alleles	Allele Digested by RE
	rs10430091	A/T	
NlaIII	rs2050927	A/T	A
NlaIII , HpyCH4V	rs4329520	A/T	T, T*
	rs4657868	A/T	
HpyCH4V	rs4658481	A/T	A
	rs6693568	A/T	
	rs860954	A/T	
	rs9431593	A/T	

* Both enzymes, NlaIII and HpyCH4V, digest the T allele.

TABLE 3: DNA samples used and genotypes

Restriction Enzyme	DNA*	SNP genotypes		
		rs2050927	rs4329520	rs4658481
HpyCH4V	NA05995		TA	TA
	NA10849		T	A
NlaIII	NA10862	AT	TA	
	NA10846	A	T	

* DNA samples were obtained from Coriell CEPH DNA collection

TABLE 4: DNA mixtures (Listed as ng DNA per reaction)

		Relative percentage unrecognized SNP allele					
		0%	2%	5%	20%	50%	100%
HpyCH4V	NA05995	0	0.6	0.6	0.6	0.6	0.6
	NA10849	0.6	29.4	11.4	2.4	0.6	0
NlaIII	NA10862	0	0.6	0.6	0.6	0.6	0.6
	NA10846	0.6	29.4	11.4	2.4	0.6	0

5 NOTE: Based on 3 pg DNA for haploid human genomic equivalent, 0.6 ng DNA is equal to 200 copies of genomic target DNA in the mixtures.

After preparation of the sample DNA mixtures, PCR cocktail was prepared according to Table 5 below (using multiplexed PCR primers as shown in Table 6) to either include no restriction endonuclease or 0.25U of restriction endonuclease per each sample reaction. PCR cocktail was aliquoted to a 96-well plate to include 7 replicates of each DNA mixture for each enzyme condition. After addition of DNA to the PCR cocktail mixtures, samples were incubated at 37°C for 1 hour to allow enzyme digestion of DNA samples and then immediately thermal cycled using standard conditions (Table 7).

TABLE 5: PCR cocktail preparation for each multiplex without DNA addition

Reagents	Final Conc	No RE N = 1 (uL)	HpyCH4V N = 1 (uL)	NlaIII N = 1 (uL)
Water	n/a	3	2.95	2.975
10xPCR Buffer (HotStar Taq Buffer)	1.25x	3.125	3.125	3.125
MgCl ₂ (25mM)	1.625mM	1.625	1.625	1.625
PCR Nucleotide Mix (for UNG use) (10mM dATP, dCTP, dGTP, dUTP)	0.2mM	0.5	0.5	0.5
F/R Primer mix (0.5uM)	0.1 μM	5	5	5

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5U/ul HpyCH4V or 10U/ul NlaIII	0.25U/rxn	--	0.05	0.025
1U/ul Uracil-DNA-Glycosylase (UDG)	1.25U/rxn	1.25	1.25	1.25
HotStar Taq (5U/uL)	2.5U/rxn	0.5	0.5	0.5
DNA - added separately	varies	10	10	10
Total volume	n/a	25	25	25

TABLE 6A: PCR Primer sequences for SNPs

SNP	Forward PCR Primer	Reverse PCR Primer
rs10430091	ACGTTGGATGCACAAGATT CTGAAACTTAG	ACGTTGGATGGCTGTTTAACTCAG CATG
rs2050927	ACGTTGGATGTTGGGTGC AGAGTAGTCATC	ACGTTGGATGTTCTAGCTTGCTTC TCCTCC
rs4329520	ACGTTGGATGATGTCCACC TCCTGCTCCAC	ACGTTGGATGGAAAGTTGTCGTGG TAGAGG
rs4657868	ACGTTGGATGCTAGCGTAC CCAATGGAATC	ACGTTGGATGCTAACCAGGAAAAG ACACCC
rs4658481	ACGTTGGATGGTGGTAGA AACAAATGTCAGC	ACGTTGGATGCTGCTAAGCATGAG AGAAAG
rs6693568	ACGTTGGATGGGCCTGTT CATTCTCAGAAA	ACGTTGGATGTGACTAGGAAATCA CACTGG
rs860954	ACGTTGGATGTAGCCTTTA GTCTTGATGCC	ACGTTGGATGCCATTCTTGTATGT TTTGTC
rs9431593	ACGTTGGATGGCCTCAGTA GTCACATAAGG	ACGTTGGATGTTGAGATCAGTGTC GGTTCC

TABLE 6B: Extend Primers

SNP	Extend Primer
rs10430091	gTGTTTAACTCAGCATGTGGGAA
rs2050927	CCTCCATCATCCTTAGC
rs4329520	GCGTGTTCTAGACTTATGC
rs4657868	cAAGACACCCCCATACATTA
rs4658481	TAAGCATGAGAGAAAGGGAAAG
rs6693568	atGAAATCACACTGGACATTTT
rs860954	GTTTTGTCTTTTCTGTATACTCATG
rs9431593	TGTTCTGACTCTCAAAAT

TABLE 7: Thermal cycling conditions

Temp.	Time	Cycles
37°C	1 hour	1
94°C	15 min	1
94°C	20 sec	45 cycles
56°C	30 sec	
72°C	1 min	
72°C	3 min	1
4°C	forever	1

Amplicon generated during PCR was genotyped with the extend primers in Table 5 using standard iPLEX™ assay and MassARRAY® technology (Jurinke, C., Oeth, P., van den Boom, D., MALDI-TOF mass spectrometry: a versatile tool for high-performance DNA analysis. *Mol. Biotechnol.* 26, 147-164 (2004); and Oeth, P. et al., iPLEX™ Assay: Increased Plexing Efficiency and Flexibility for MassARRAY® System through single base primer extension with mass-modified Terminators. SEQUENOM Application Note (2005), both of which are hereby incorporated by reference).

Results

Digestion of DNA with both restriction enzymes allowed detection of minor alleles when they were present at ratios as low as 2% heterozygous DNA. This is in contrast to undigested DNA samples where minor alleles were only reliably detected when present at ratios of 20% heterozygous DNA and higher. When allele peak area ratios are considered, the effect of restriction endonuclease digest is even more apparent. HpyCH4V digested samples showed minor allele peak area ratios of 0.35-0.45 in 2% heterozygous DNA mixtures, while minor allele peak area ratios of 2% heterozygous DNA mixtures were at background levels without enzyme digestion (Figure 1). While the increases in allele peak area ratio were not as high when using the NlaIII restriction endonuclease, the results were similar (Figure 2). Example screen shots of the mass spectrum in 2% heterozygous DNA mixtures with and without HpyCH4V (Figure 3) or NlaIII (Figure 4) are shown below.

Optimization studies

Initial optimization studies for enzyme concentration and pre-PCR incubation time of HpyCH4V digestion were performed using 5% heterozygous DNA mixtures (0.6 ng heterozygous DNA, 11.4 ng homozygous DNA). Based on these experiments, maximal peak area ratios were obtained with incubation times as short as 5 minutes and 0.25U HpyCH4V enzyme.

Example 2: Restriction Endonuclease Enhanced Polymorphic Sequence Detection Using Tfil

A similar experiment was performed as described in Example 1 using a different restriction endonuclease, Tfil. In this experiment, the Tfil restriction endonuclease selectively recognized and cleaved the 'C' allele of the 'C/T' SNP, rs4487973. The SNP rs4487973 occurs in the following genomic sequence on chromosome 1: CACACAGTTAGGATT[C/T]ACCTGAGCTTGTCCC. For these studies, two CEPH DNA samples, one homozygous 'C' and the other heterozygous 'C/T' for the rs4487973 SNP, were mixed in varying ratios to generate DNA mixtures containing 0%, 1%, 2.5%, 10%, 50% of the rs4487973 'T' allele. The Tfil restriction endonuclease was either added or not added to each mixture to determine the endonucleases' effect on detecting the polymorphic sequence. Of the mixtures not digested with Tfil enzyme, the rs4487973 'T' allele was detected in the 10%, and 50% 'T' allele mixtures, but not the 0%, 1%, and 5% 'T' allele DNA mixtures. However, of samples digested with Tfil enzyme, the rs4487973 'T' allele was detectable in 1%, 5%, 10% and 50% 'T' allele mixtures. These results indicate the utility of this method to improve detection of polymorphic alleles present at low relative concentrations in a sample.

Example 3: Fetal Identifiers, Sex Test And Copy Number Determination*Selection of SNPs*

Analysis of paternally-inherited alleles in clinical samples and correlation with Y-chromosome frequency in male fetuses was performed with a total of 16 SNPs. SNP assays for analysis of clinical samples were multiplexed as 8-plexes. All SNPs had a minor allele frequency (maf) of ~0.4 in all ethnic groups and were unlinked.

For performance evaluation of a universal Fetal Identifier panel that can be multiplexed with disease-specific markers, a new panel of 87 A/T SNPs with a pan-ethnic maf >0.4 was selected and multiplexed into 16-plexes.

Method of SNP analysis

Analysis of SNPs in maternal buffy coat and maternal plasma was performed using the iPLEX™ assay and MassARRAY® technology. In brief, the target region surrounding the SNP is first amplified by PCR. Subsequently an oligonucleotide primer is annealed to the PCR product and is extended allele-specifically by a single nucleotide using a mixture of 4 terminator nucleotides and a DNA polymerase. The extension products are transferred to a miniaturized chip array and are analyzed by MALDI-TOF Mass Spectrometry. Determination of the molecular mass of extension products allows unambiguous identification of the SNP allele present in the sample.

The peak area ratio of mass signals allows the estimation of the relative abundance of the alleles in a given sample. Figure 5A provides an overview of the assay used for SNP analysis.

Clinical Samples

5 The total sample set consisted of 35 paired blood/plasma samples from pregnant Caucasian woman (nine 1st trimester; twelve 2nd trimester; fourteen 3rd trimester).

The subset of samples used for correlation of Y-chromosome frequency and paternally-inherited alleles in maternal plasma consisted of 19 samples of pregnant Caucasian woman carrying a male fetus.

DNA extraction

DNA extraction was performed from 1ml of maternal plasma using the Qiagen® MinElute kit for fetal genotyping.

10 DNA extraction from frozen blood (minus plasma) was performed from 4ml using Qiagen's PureGene kit for maternal genotyping.

Results

An assay targeting sequence differences in the Amelogenin region on the X and Y chromosome was used to assess the relative amount of fetal DNA extracted from plasma of pregnant woman carrying a male fetus. Details of the AMG assay are depicted in Figures 8A-8C. X and Y-specific sequences can be discriminated by sequence specific iPLEX extension products and their respective mass signals. The peak area ratio of the extension products allows estimation of the relative amount of fetal DNA, because the Y-specific sequences represent 50% of the total fetal DNA contribution.

25 Sixteen of nineteen (84%) plasma samples with a male fetus showed a Y-chromosome frequency of higher than 5%, indicating presence of at least 10% fetal DNA in the extracted DNA.

Figure 6 depicts typical performance results for a qualified fetal identifier. Here the ability of the SNP assay to estimate the quantity of fetal DNA in the background of maternal DNA was verified for a total of 1700 copies and a total of 170 copies using genomic DNA mixtures. Note that the standard deviation of the estimate of fetal DNA increases due to the significant influence of the sampling error at low copy numbers

30 Table 8 provides a list of SNPs that were multiplexed at 10+ plexing level and passed all phases of the validation.. The following shows the validation scheme, performance criteria and model system used to qualify multiplex SNP assays for their utility in identifying the presence for fetal DNA.:

Phase I

Step 1: Initial Fetal Identifier (FI) screening parameters

- FI's are multiplexed from pool of 87 A/T SNPs (mass difference 56 Da)
- Genotyping of control DNAs (CEPH populations)

5 Step 2: Advance screening criteria

- Reproducibility of genotyping calls in 4 replicates
- Unambiguous genotype data (assay shows no interfering or unpredicted mass signals)
- Allelic skew in heterozygous DNAs
- Variance of allelic ratio in heterozygous DNAs

10

Step 3: Replex successful SNPs and repeat Phase 1 screening to generate multiplexes of 10+ SNPs

Multiplexed SNPs passing Phase I test criteria are tested in Phase II

15

Phase II

Step 1: Mixtures of Genomic DNA are used for assessing FI reliability

- Mix Mother: 2000 copies of DNA1
- Mix 10%: 3600 copies DNA 1 / 400 copies of DNA 2
- Mix 20%: 1600 copies DNA 1 / 400 copies of DNA 2

20

Analysis of allele frequency variation in 4 mixture series and 8 replicate measurements. Sensitivity and specificity are calculated for the detection of low copy number allele in background of high copy number allele

25 Multiplexed SNPs passing Phase II test criteria are tested in Phase III

Phase III

Step 1: Various DNAs are mixed to emulate different maternal-fetal combinations

- Plate 1: 3600 copies DNA maternal / 400 copies DNA fetal
- Plate 2: 1600 copies DNA maternal / 400 copies DNA fetal

30

Each plate contains 88 sample mixtures, 4 positive and 4 negative controls. Analysis of allele frequency variation in 4 mixture series, where sensitivity and specificity are calculated for the detection of low copy number allele in background of high copy number allele

Application of this assay panel to a model system for the detection of fetal DNA in maternal background showed that paternally-inherited fetal alleles can be detected with a sensitivity of 95% at 100% specificity if the sample preparation method can enrich the relative amount of fetal DNA to 20%. In Table 8, the minor allele frequency (MAF) for each SNP from different ethnic populations is provided. The ethnic populations are defined by the HapMap Project, where CEU represents individuals of Northern and Western Europe descent, HCB represents Han Chinese in Beijing, JAP represents Japanese in Tokyo, and YRI represents the Yoruba in Ibadan, Nigeria.

TABLE 8

SNP	MAF CEU	MAF HCB	MAF JAP	MAF YRI
rs11166512	0.43	0.41	0.50	0.49
rs11184494	0.50	0.40	0.48	0.50
rs11247894	0.43	0.39	0.32	0.44
rs12089156	0.46	0.49	0.44	0.43
rs12125888	0.40	0.43	0.48	0.43
rs12136370	0.42	0.48	0.42	0.48
rs12143315	0.40	0.42	0.42	0.42
rs12759642	0.39	0.48	0.48	0.42
rs156988	0.46	0.40	0.45	0.41
rs2050927	0.44	0.50	0.41	0.49
rs213624	0.48	0.44	0.40	0.34
rs2454175	0.46	0.48	0.43	0.40
rs4329520	0.45	0.43	0.40	0.44
rs4487973	0.47	0.43	0.44	0.40
rs454782	0.48	0.40	0.41	0.46
rs4648888	0.33	0.30	0.33	0.46
rs635364	0.49	0.40	0.46	0.43
rs660279	0.41	0.49	0.50	0.39
rs6687785	0.48	0.46	0.48	0.44
rs7551188	0.46	0.49	0.45	0.46
rs9431593	0.41	0.43	0.49	0.40

A multiplexed panel of 16 SNPs was analyzed with maf>0.3 in the same maternal plasma DNA extraction and established a baseline of maternal genotypes by analyzing DNA from PBMCs. Using the maternal genotype information, paternally-inherited alleles were identified in plasma samples and estimated the amount of fetal DNA from the peak area ratio of extension products representing paternally-inherited fetal alleles and maternal alleles.

The AMG XY frequency was then compared with the allele-frequency of paternally-inherited fetal alleles in informative SNPs. This comparison revealed that samples with a positive Y-frequency of 10% (used as a Limit-of-quantitation threshold) or more have significantly higher

differences between maternally and paternally-inherited fetal allele-frequencies (p-value < 0.001; Fishers' exact test). This data suggests that Fetal Identifiers can be used as a non-gender specific approach for identification of the presence of fetal DNA. Figure 7 exemplifies those results.

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TABLE 9

Multiplex	Primer Name	Amplification primer	Amplification primer	Extend Primer sequence
MP1	rs75511 88	<u>ACGTTGGATGATCCCTGGTTCCTT</u> CCTTAG	<u>ACGTTGGATGGAGCCTCTCAGTG</u> TCTATAC	GGACAGATTCTGGGAC
MP1	rs11247 894	<u>ACGTTGGATGATCCTAGATAGCC</u> CAAAGCC	<u>ACGTTGGATGGGAGGAAAGAGAA</u> GATTGTG	CCAAAGCCCAAGAAATTCA
MP1	rs66877 85	<u>ACGTTGGATGATGCTGTAAAGAG</u> CCTCAAC	<u>ACGTTGGATGTTCTCCTCTGACC</u> TGCTTTC	CCTCAACAGTACACTTAATC
MP1	rs44879 73	<u>ACGTTGGATGTCAGAGAGTGACA</u> AGACCTG	<u>ACGTTGGATGGAATGCATGCCAA</u> CTTAGGG	cAGGTCACACAGTTAGGATT
MP1	rs46488 88	<u>ACGTTGGATGCAGAGAGTCCCCT</u> GTTATTG	<u>ACGTTGGATGTGCCCAGACCAGA</u> GAGGTCA	aTGGACCTTCGGAAAAGGATA
MP1	rs12089 156	<u>ACGTTGGATGGCTACATACTATGT</u> GGTCTC	<u>ACGTTGGATGCCTGCTGGCAACA</u> AATCTTC	TACTATGTGGTCTCAACTATA T
MP1	rs20509 27	<u>ACGTTGGATGTTCTAGCTTGCTTC</u> TCCTCC	<u>ACGTTGGATGTTGGTGCAGAGT</u> AGTCATC	TGCTTCTCCTCCATCATCCTT AGC
MP1	rs12125 888	<u>ACGTTGGATGCAACATCCTGTAC</u> ATCACTC	<u>ACGTTGGATGAGACAATTTCTGT</u> CCTCTGG	TACATGACTATCTCCTCCCTT AGGT
MP1	rs12143 315	<u>ACGTTGGATGACAGGCATGAGCC</u> ATCTTAC	<u>ACGTTGGATGTGCCATTGGTACA</u> GTCACTC	CCATCTTACCCAGCCTCTTTTC TTCAA
MP1	rs21362 4	<u>ACGTTGGATGTAGGTCAAGCCAA</u> GGCCTC	<u>ACGTTGGATGTCCACCCAGGA</u> GCAGCCA	gGCCAAGGCCCTCGGAGTCTG AACAGTT
MP1	SRY_5- ib	<u>ACGTTGGATGAGCATCTAGGTAG</u> GTCTTTG	<u>ACGTTGGATGAGCAACGGGACC</u> GCTACAG	cGTTACCCGATTGTCTCTAC
MP2	rs66027 9	<u>ACGTTGGATGTTTCAGCAACCACT</u> CTGAGC	<u>ACGTTGGATGTGCCCGTAAGTAG</u> GAGAGTG	CTTGATGTGCTTCCCTG
MP2	rs63536 4	<u>ACGTTGGATGGAAATTTCTGGATT</u> ACTGGC	<u>ACGTTGGATGAGAGACTCCATTT</u> GTTTGGG	TGGATTACTGGCAAAAGAC
MP2	rs94315 93	<u>ACGTTGGATGTTGAGATCAGTGT</u> CGGTTCC	<u>ACGTTGGATGGCCTCAGTAGTCA</u> CATAAGG	TGTTCTGACTCTCAAAAT

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MP2	rs11166 512	<u>ACGTTGGATGCTTCATCCACTATA</u> TCCACC	<u>ACGTTGGATGTGACCAGATGTTG</u> GATTAG	CCACTATATCCACCTTTTCT
MP2	rs43295 20	<u>ACGTTGGATGGAAAGTTGTCGTG</u> GTAGAGG	<u>ACGTTGGATGATGCCACCTCCT</u> GCTCCAC	GCGTGGTTCTAGACTTATGC
MP2	rs45478 2	<u>ACGTTGGATGCTGTTAAGATGCC</u> AACTCCC	<u>ACGTTGGATGCTGCTTCCCTCATT</u> GCTCTG	AACTCCCATATTAGTCCACAG
MP2	rs12136 370	<u>ACGTTGGATGGAGTAGTTCTTTG</u> CAGTAAGC	<u>ACGTTGGATGCTCCTGGAAACA</u> GCAAAAG	gGCAGTAAGCTATTCTTGGGG
MP2	rs12759 642	<u>ACGTTGGATGATTCTTCCTGGGA</u> CTCAGAC	<u>ACGTTGGATGGGAAATACCAGCA</u> ACCACAG	caTCGGGATTCCCTGAACAAA A
MP2	rs11184 494	<u>ACGTTGGATGAGCTGGCCATGTT</u> TATTTGAC	<u>ACGTTGGATGGCCAATCTATGAA</u> GAATTAC	ATTTGACTTTCCCTACTCCTTAA C
MP2	rs24541 75	<u>ACGTTGGATGGGAATCAGACCTG</u> TAAACAC	<u>ACGTTGGATGGCCAGCAGGAC</u> ACTTTTAT	cCTCAAGGATTGGAATTAGA GT
MP2	rs15698 8	<u>ACGTTGGATGAAAGCTCTGTGAT</u> GCGTCTC	<u>ACGTTGGATGGAAAGGCTATGT</u> AAGGAGG	tCGTCTCGGTCTTCCCTTTTCA CTT

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TABLE 10

Multiplex	SNP_ID	Amplification primer	Amplification primer	Extend Primer sequence
W1	rs10793675	<u>ACGTTGGATGAAGAGATGAGACA</u> GACTGGG	<u>ACGTTGGATGCTCTGTATTTATAG</u> CTTTC	AACGGCTCAACAGTT
W1	rs1829309	<u>ACGTTGGATGATCTCTGAGTTGA</u> CACCACC	<u>ACGTTGGATGTTCTTAATCAGGAG</u> AGACCG	TTGCTTTGGGGAGCAG
W1	rs660279	<u>ACGTTGGATGTTTCAGCAACCACT</u> CTGAGC	<u>ACGTTGGATGCCCCGTAAGTAG</u> GAGAGTG	CTTGATGTGCTTCCCTG
W1	rs635364	<u>ACGTTGGATGGAAATTTCTGGATT</u> ACTGGC	<u>ACGTTGGATGAGAGACTCCATTTG</u> TTTGGG	TGGATTACTGGCAAAGAC
W1	rs9431593	<u>ACGTTGGATGTTGAGATCAGTGT</u> CGGTTCC	<u>ACGTTGGATGGCCTCAGTAGTCA</u> CATAAGG	TGTTCTGACTCTCAAAAT
W1	rs11166512	<u>ACGTTGGATGCTTCATCCACTATA</u> TCCACC	<u>ACGTTGGATGTGACCAGATGTTG</u> GATTAG	CCACTATATCCACCTTTTCT
W1	rs4329520	<u>ACGTTGGATGGAAAGTTGTCGTG</u> GTAGAGG	<u>ACGTTGGATGTCCACCTCCTG</u> CTCCAC	GCGTGGTTCTAGACTTATGC
W1	rs454782	<u>ACGTTGGATGCTGTTAAGATGCC</u> AACTCCC	<u>ACGTTGGATGCTTTCCTCATT</u> GCTCTG	AACTCCCATATTAGTCCACA G
W1	rs12136370	<u>ACGTTGGATGGAGTAGTTCTTTG</u> CAGTAAGC	<u>ACGTTGGATGCTCCTGGAAAACA</u> GCAAAAG	gGCAGTAAGCTATTCTTGGG G
W1	rs12759642	<u>ACGTTGGATGATTCTTCCTGGGA</u> CTCAGAC	<u>ACGTTGGATGGGAAATACCAGCA</u> ACACAG	caTCGGGATTCCCTGAACAA AA
W1	rs11184494	<u>ACGTTGGATGAGCTGGCCATGTT</u> TATTTGAC	<u>ACGTTGGATGGCCAATCTATGAAG</u> AATTAC	ATTGACTTTTCTTACTCCTTA AC
W1	rs2454175	<u>ACGTTGGATGGGAATCAGACCTG</u> TAAACAC	<u>ACGTTGGATGGCCCAGCAGGACA</u> CTTTTAT	cCTTCAAGGATTGGAATTAGA GT
W1	rs1452628	<u>ACGTTGGATGGCTTGTCCTTTGTT</u> GTGTGG	<u>ACGTTGGATGGTCAAGCAAAGG</u> CTTCAAG	acatAGTTATTCTTAGGGCTTC TC
W1	rs15698	<u>ACGTTGGATGAAAGCTCTGTGAT</u>	<u>ACGTTGGATGGAAAGGGCTATGT</u>	tCGTCTCGGTCCTTCTCTTTTC

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	8	GCGTCTC	AAGGAGG	ACCT
W1	rs45704 30	ACGTTGGATGACCCGAGCCAATC AGGTATC	ACGTTGGATGGCACATGGAGATG AATGGTC	GGTATCATAAGATACCTATG ATGTC
W1	rs12062 414	ACGTTGGATGTGCGTCAACCTTT CCAGTTC	ACGTTGGATGGGAAAGTCCTCGA CTGTTTG	ggaaTTTCCAGTTCTATTCCA GCCTC
W1	rs75453 81	ACGTTGGATGCCAGTCAAGCTAA GGACAAA	ACGTTGGATGGTGAGCACAACTG TGTTCTA	tccCTGAATGACAAAAGGGGA AGATA
W1	rs64276 73	ACGTTGGATGGGACTAAAACAGG GCCAAAC	ACGTTGGATGGTCTCTCTAGTACT AGTAAC	ccctcGCCAAAACTTAGACCAAG GACAAC
W1	rs10802 761	ACGTTGGATGTCTTCTAAATGTA GTTATG	ACGTTGGATGGGATGAGGTTTTG ACTAAGC	AGTTATGAAATAAGTTTTTATT CATTTAC

W2	rs64244 9	ACGTTGGATGCCAAAACCATG CCCTCTG	ACGTTGGATGAGATTGCCTCTCCA TGTGAC	CCTCTGCCCTCCCCTA
W2	rs48394 19	ACGTTGGATGCTGCCGCATCCCT TCACAA	ACGTTGGATGATGTGTTTGTGGCC ACTTCC	CCTTCACAAAGCCGA
W2	rs93241 98	ACGTTGGATGAAAGGCCTACTGT TTGCTGG	ACGTTGGATGCAAAATATGTGTGA ATCAGC	cGTTTGTGTTGGAAGCCT
W2	rs11926 19	ACGTTGGATGGCTCAACTCTGAA CCAATCG	ACGTTGGATGCCAGGAATGGGCA TGTGTTT	TGGCCAGAAAGAGGAG
W2	rs46578 68	ACGTTGGATGCTAACCAGGAAAA GACACCC	ACGTTGGATGCTAGCGTACCCCAAT GGAATC	AGACACCCCCATACATTA
W2	rs64268 73	ACGTTGGATGTAATCAGGGCTG CCTTCTC	ACGTTGGATGAAGTGCTAGGGTT ACAGGTG	cccTGCCCTTCTCTTCCAA
W2	rs43898 1	ACGTTGGATGTGTGCAAAATTGGC TAACAT	ACGTTGGATGGAACATTGGTATTT AAACTC	ATGGACCACAAAAAACTTA
W2	rs12125 888	ACGTTGGATGAGACAATTTCTGTC CTCTGG	ACGTTGGATGCAACATCCTGTACA TCACTC	TCTGTCTCTGGTATCCTCT
W2	rs31286 88	ACGTTGGATGATCAAGAGGAAAA TGGACAG	ACGTTGGATGGATTTACTCAACTC TCTGGG	cAAAATGGACAGAAAGTTGAA
W2	rs49873 51	ACGTTGGATGGTGCATGGGCTCA TCTAGAC	ACGTTGGATGCCAAACAGGGCCA ATGGTAG	gCATCTAGACACATTTTGTGC
W2	rs66929	ACGTTGGATGCTATTCCCTCCTCA	ACGTTGGATGATTAAAGATGGGTAG	tccAAGAGCATTTTTCCTCTTC

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	11	AAGAGC	TAAAG	
W2	rs66846 79	ACGTTGGATGATGTTACTTGCCT TGGCCC	ACGTTGGATGTCCTTAAGGTGTCTC CCTCTG	ggaCCACTGAGGAGATACAC TA
W2	rs43208 29	ACGTTGGATGGGTTCTATGGCTTT GGTGAG	ACGTTGGATGTGCTAGACACTTTA ACTGCC	ggtcACCTCTTTTTCATAACAGG A
W2	rs46584 81	ACGTTGGATGCTGCTAAGCATGA GAGAAAG	ACGTTGGATGGTGTAGAAACAA ATGTCAGC	atacGCATGAGAGAAAAGGGAA AG
W2	rs37684 58	ACGTTGGATGCCAAATGTCTTAGT TACAAAG	ACGTTGGATGGAGTTTATGTAATG TCAAC	CTTAGTTACAAAGAAAAATTGT GAG
W2	rs86095 4	ACGTTGGATGTAGCCTTTAGTCTT GATGCC	ACGTTGGATGCCATTCTTGTATGT TTTGTC	TCTTGATGCCCTTACAAAAATAA ATAT
W2	rs10453 878	ACGTTGGATGGAGGAGCTAACAA GTAGGAC	ACGTTGGATGGGATATGAATTAC AACAGAG	AAACAAATCCTCCTTTCTTTT AATTC
W2	rs10753 912	ACGTTGGATGGAGATTATATGTCT CTTTAA	ACGTTGGATGATTCTTCTAACCTTT AGGC	GAGATTATATGTCTCTTTTAAT ATTGTC
W2	rs16379 44	ACGTTGGATGCTAATGCCTCCTTT TCTTCC	ACGTTGGATGAATAGCAAACAACA GGTGGG	cccccATATCATTTTGCAATTGC ATGGTT
W2	rs48392 82	ACGTTGGATGGAATCCTGGCAGC TCATTAG	ACGTTGGATGTGGTTTCACATGA GTC TTGC	gatgTCTCTTAAAGAGCAAAAA GCTAAG

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TABLE 11

SNP ID	Amplification primer	Amplification primer	Extend Primer sequence
rs429235	<u>ACGTTGGATGCTTACCCATGCTAAGT</u>	<u>ACGTTGGATGAAGATTATGGAGGCCA</u>	TCCCACCTCCTCCGTGC
7	CCTG	GGAG	
rs994099	<u>ACGTTGGATGCTCTGGAATGCCCCCTTC</u>	<u>ACGTTGGATGGAGCATATTCTGAGGA</u>	GAAAGTACCTCCCATGC
5	TTAG	TGGG	
rs112244	<u>ACGTTGGATGTTCCCTTTCTTCCAGAT</u>	<u>ACGTTGGATGGTATATAGCTGGGCAT</u>	GCCCAGGGTGAATTAAA
3	GGG	GGTG	
rs107616	<u>ACGTTGGATGCTCTGTCACTGACTTC</u>	<u>ACGTTGGATGTACTCAGAGGAACCTG</u>	TGACTTCATGGGACCTCA
70	ATGG	AGTC	
rs256064	<u>ACGTTGGATGGGCTCTAAGCCATTCT</u>	<u>ACGTTGGATGGCCTTTCAAAGCCACA</u>	tTCCCCCTGTCCCTCTGCC
3	TCTG	TCTC	
rs131340	<u>ACGTTGGATGGGAGTTCTTATCCATA</u>	<u>ACGTTGGATGGGAAGCTTCTGGGAC</u>	TTTTCTGTGCTTTTTCCTC
04	CTATG	TTAAT	
rs110774	<u>ACGTTGGATGAACCTCCCATTTCTCCC</u>	<u>ACGTTGGATGTGGACCCAGTCAAGA</u>	gaTCCCTGTGTGGTAATGC
42	TGTG	AAGTC	
rs492417	<u>ACGTTGGATGGGTTTCATTCCCAACA</u>	<u>ACGTTGGATGCAGCCTATGTATGGAA</u>	AATGCCACATTCTTGATTGC
6	AGTC	ACAG	
rs600595	<u>ACGTTGGATGTTAAGCACTGCCTGTA</u>	<u>ACGTTGGATGGGCAGTGAACCTTGT</u>	ggaAGTGTGCTAGACGCTGC
5	TCCC	CTAC	
rs989309	<u>ACGTTGGATGAACCCACTATACCCCA</u>	<u>ACGTTGGATGACACACACATTCTGCT</u>	cccAGGCCCAAGTCTTCTGC
6	ACTC	GAGG	
rs724182	<u>ACGTTGGATGTAACCTTGATTACTGGC</u>	<u>ACGTTGGATGCCAGGTGTGTCTCAA</u>	gggACTGGCACTGCCCCATA
3	ACTG	ATTC	
rs602643	<u>ACGTTGGATGCTCAAAGGTGCCAATC</u>	<u>ACGTTGGATGCTGATGGCTTGCTTTG</u>	aCACAGGCCCATAAACTATAA
6	ACTC	ATTC	C
rs792688	<u>ACGTTGGATGCTGAACAAGACCTTGA</u>	<u>ACGTTGGATGGTTGAGACTGCCAGC</u>	ggACAAAGACCTTGAAGTTGATA
7	AG	TATTC	
rs178113	<u>ACGTTGGATGTACAACTGGATGCTGA</u>	<u>ACGTTGGATGTTAGACATGAGGCC</u>	aAAAAAGAGAGTTGAAGTTTAG
18	GTAG	CAAG	G
rs578381	<u>ACGTTGGATGTGTTTCTTGAGGCTC</u>	<u>ACGTTGGATGAACAGGTCTGACATCT</u>	gttcGGCTCTCTGTGTCTGTGT
	TCTG	GTGG	

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rs978055	<u>ACGTGGGATGTTCC</u> AAGATCCCCCTT TTCC	<u>ACGTGGGATGGG</u> CATAAAGCCTTACA CCTC	gaaccTTTACTTGCTTCCTGTTG C
rs176275	<u>ACGTGGGATGTGG</u> ATCTTACCCCTAT TCAG	<u>ACGTGGGATGTTG</u> GTGTGACCAAGAC CTAC	gTCAGATAAAGTCTGAGTTTCAT TG
rs288776	<u>ACGTGGGATGCTCT</u> GATGGTATGTAGA GAGG	<u>ACGTGGGATGGGG</u> CAAAATTTCCATTT GTG	ATGGTATGTAGAGAGGTAAAT TGC
rs770291	<u>ACGTGGGATGTCCC</u> ATAGTCTGATCC CTAC	<u>ACGTGGGATGGAT</u> GTTCTCCAAAGTA GAAG	ggagaATCCCCTACCTGATGTTTT GC
rs499849	<u>ACGTGGGATGCCA</u> GGAATTGGGATAT GGAC	<u>ACGTGGGATGCCA</u> AGCTTCAAATCAA TGTC	tCTTACCACATAAAAACTATCA GTAT
rs930601	<u>ACGTGGGATGGCC</u> TGGGAAAATGATT GGAC	<u>ACGTGGGATGGT</u> GGGAGATATTGTTA AGGC	AATATTTTGAGATAAAATTGTAG ATGC

The entirety of each patent, patent application, publication and document referenced herein hereby is incorporated by reference. Citation of the above patents, patent applications, publications and documents is not an admission that any of the foregoing is pertinent prior art, nor does it constitute any admission as to the contents or date of these publications or documents.

Modifications may be made to the foregoing without departing from the basic aspects of the invention. Although the invention has been described in substantial detail with reference to one or more specific embodiments, those of ordinary skill in the art will recognize that changes may be made to the embodiments specifically disclosed in this application, yet these modifications and improvements are within the scope and spirit of the invention.

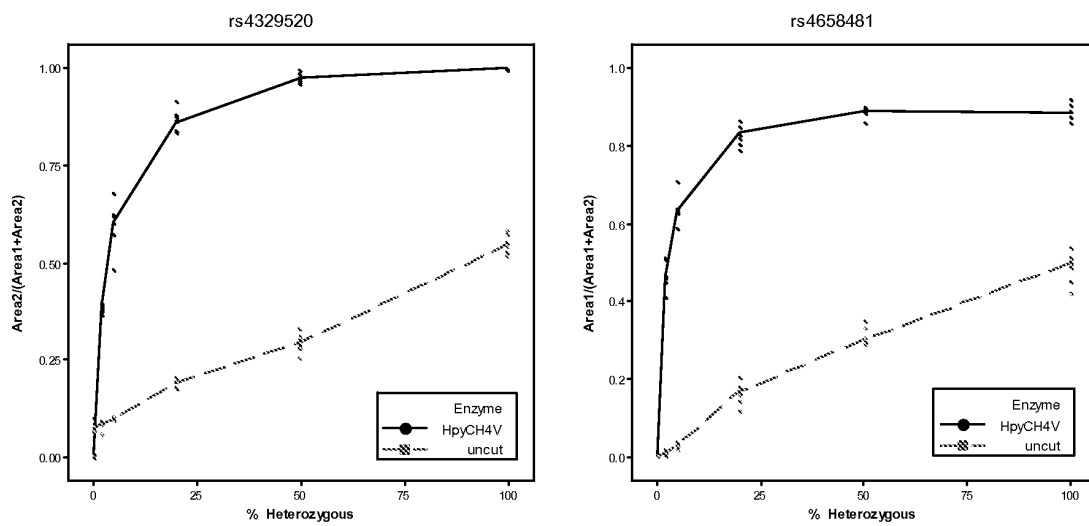
The invention illustratively described herein suitably may be practiced in the absence of any element(s) not specifically disclosed herein. Thus, for example, in each instance herein any of the terms “comprising,” “consisting essentially of,” and “consisting of” may be replaced with either of the other two terms. The terms and expressions which have been employed are used as terms of description and not of limitation, and use of such terms and expressions do not exclude any equivalents of the features shown and described or portions thereof, and various modifications are possible within the scope of the invention claimed. The term “a” or “an” can refer to one of or a plurality of the elements it modifies (e.g., “a reagent” can mean one or more reagents) unless it is contextually clear either one of the elements or more than one of the elements is described. The term “about” as used herein refers to a value within 10% of the underlying parameter (i.e., plus or minus 10%), and use of the term “about” at the beginning of a string of values modifies each of the values (i.e., “about 1, 2 and 3” is about 1, about 2 and about 3). For example, a weight of “about 100 grams” can include weights between 90 grams and 110 grams. Thus, it should be understood that although the present invention has been specifically disclosed by representative embodiments and optional features, modification and variation of the concepts herein disclosed may be resorted to by those skilled in the art, and such modifications and variations are considered within the scope of this invention.

Embodiments of the invention are set forth in the claims that follow.

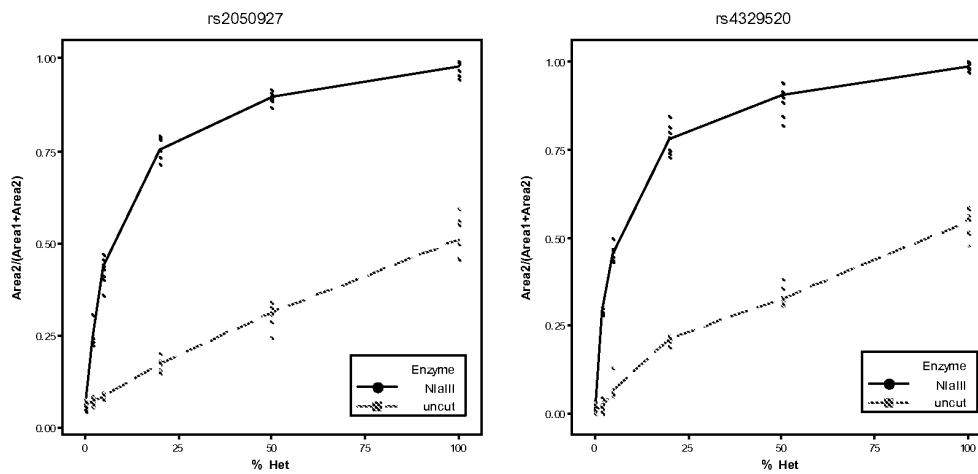
What is claimed is:

1. A method for detecting the presence or absence of a plurality of target alleles at a plurality of polymorphic loci in a sample, wherein the sample contains nucleic acid, which comprises:
 - a) cleaving nucleic acid comprising a plurality of non-target alleles at the plurality of polymorphic loci with a single cleavage agent that recognizes the non-target alleles, wherein the cleavage agent is a non-thermostable restriction endonuclease;
 - b) amplifying uncleaved nucleic acid but not cleaved nucleic acid; and
 - c) analyzing the amplification products of step b) to determine the presence or absence of a plurality of target alleles, wherein the target alleles are of paternal origin and the non-target alleles are of maternal origin.
2. The method of claim 1, wherein the sample is from a pregnant female or a female suspected of being pregnant.
3. The method of claim 1, wherein 60 or more polymorphic loci are assayed.
4. The method of claim 1, wherein 10 or more target alleles are detected.
5. The method of claim 1, wherein the non-thermostable restriction endonuclease is HpyCH4V.
6. The method of claim 1, wherein the cleavage agent is not active during step b).
7. The method of claim 1, wherein the target allele concentration is less than 4% relative to the non-target allele concentration prior to cleaving and amplifying the nucleic acid.
8. The method of claim 1, wherein the polymorphic loci are selected from the group consisting of the polymorphisms rs10430091, rs2050927, rs4329520, rs4657868, rs4658481, rs6693568, rs860954 and rs9431593.

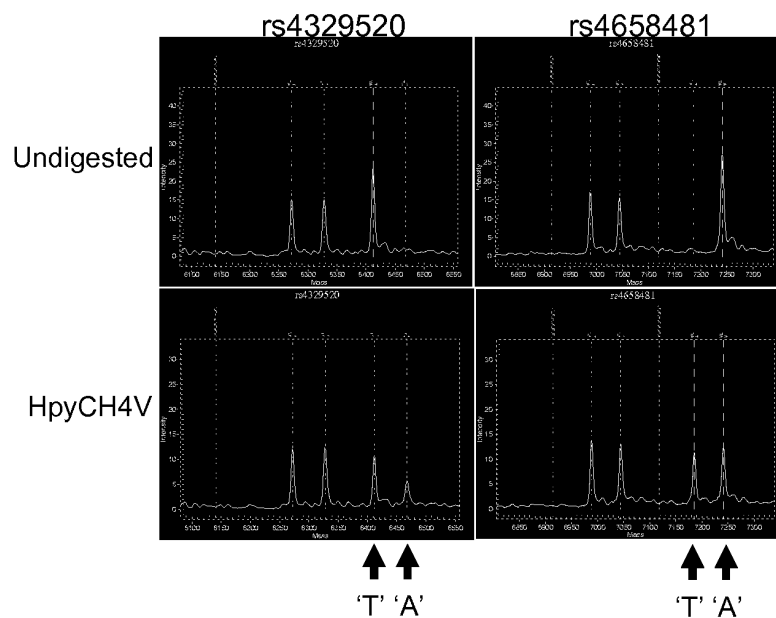
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FIGURE 1**HpyCH4V digest**

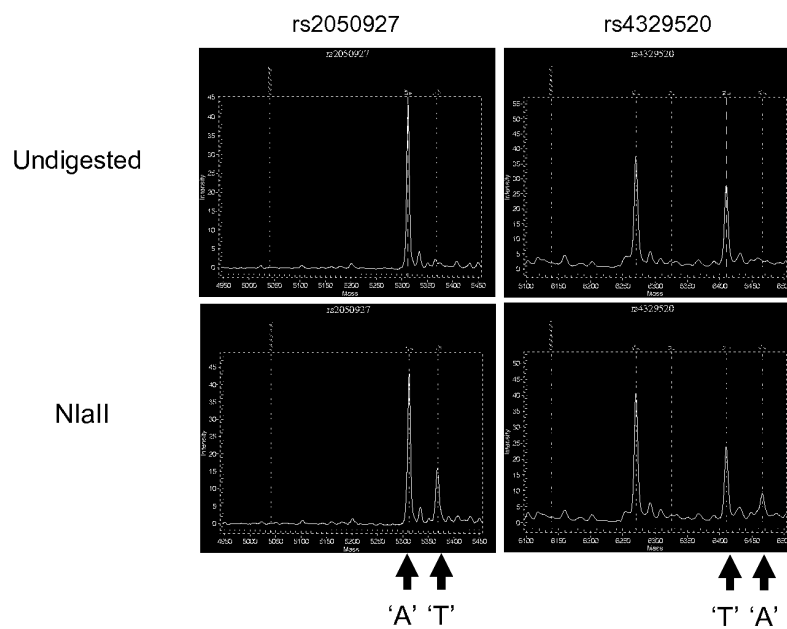
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FIGURE 2**NlaIII digest**

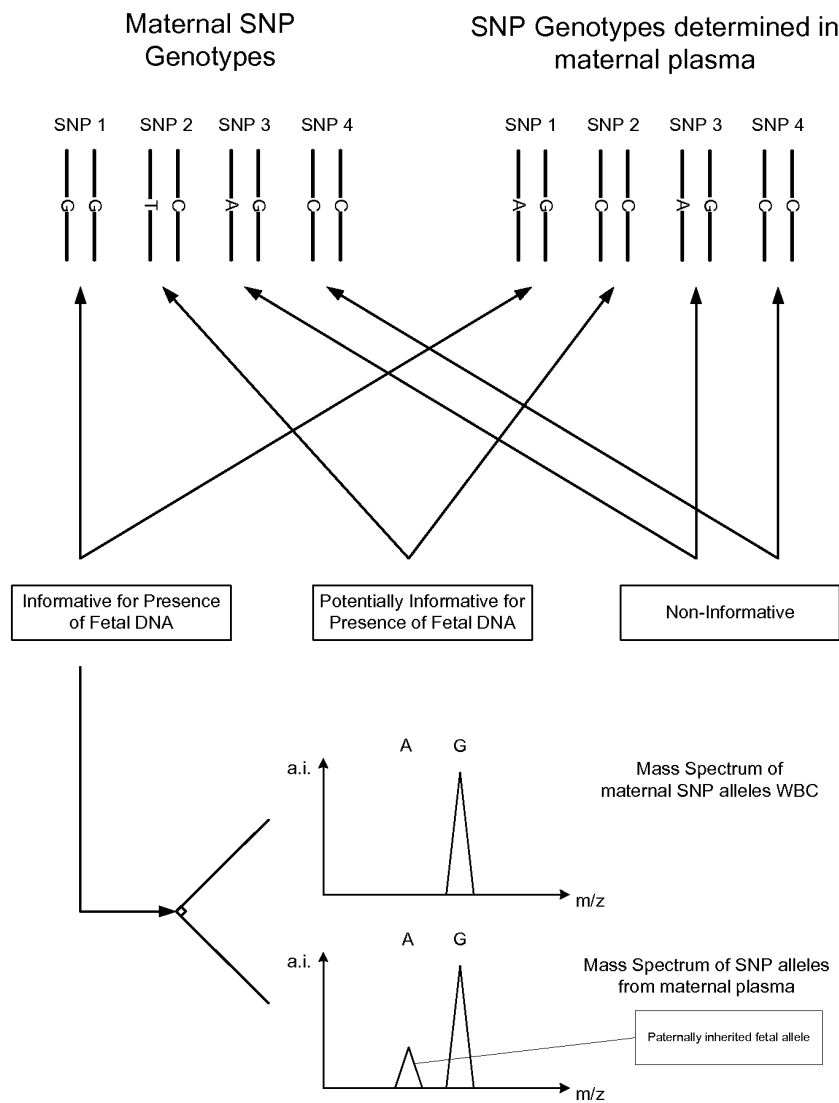
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FIGURE 3**HpyCH4V screenshots**

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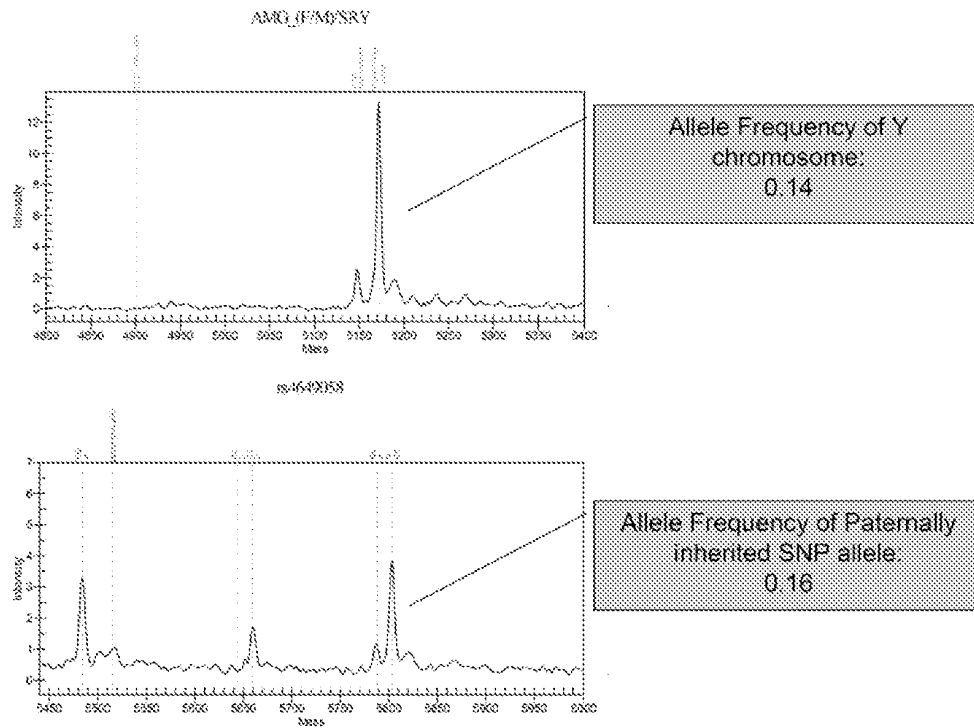
FIGURE 4**NlaIII screenshots**

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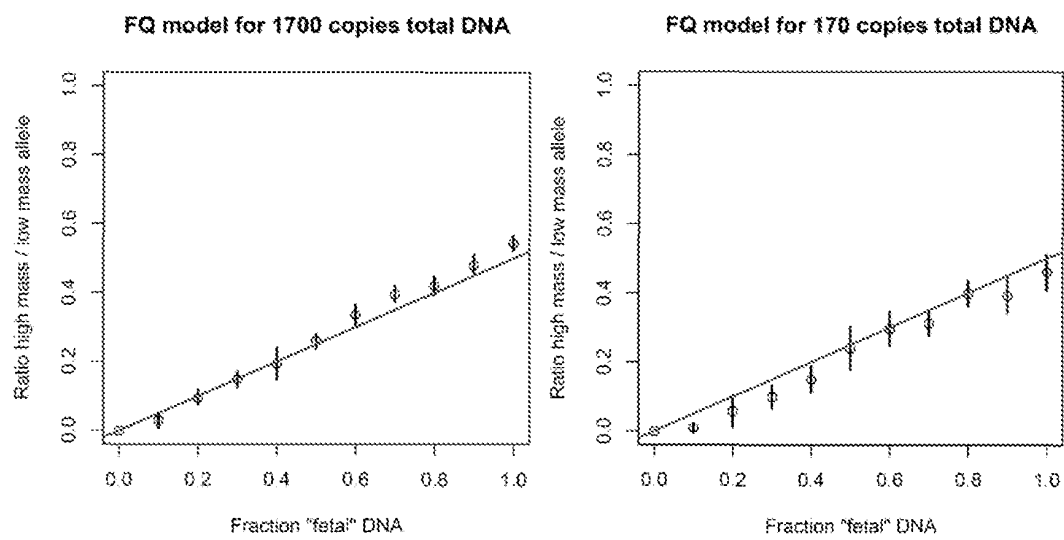
FIGURE 5A

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FIGURE 5B



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FIGURE 6

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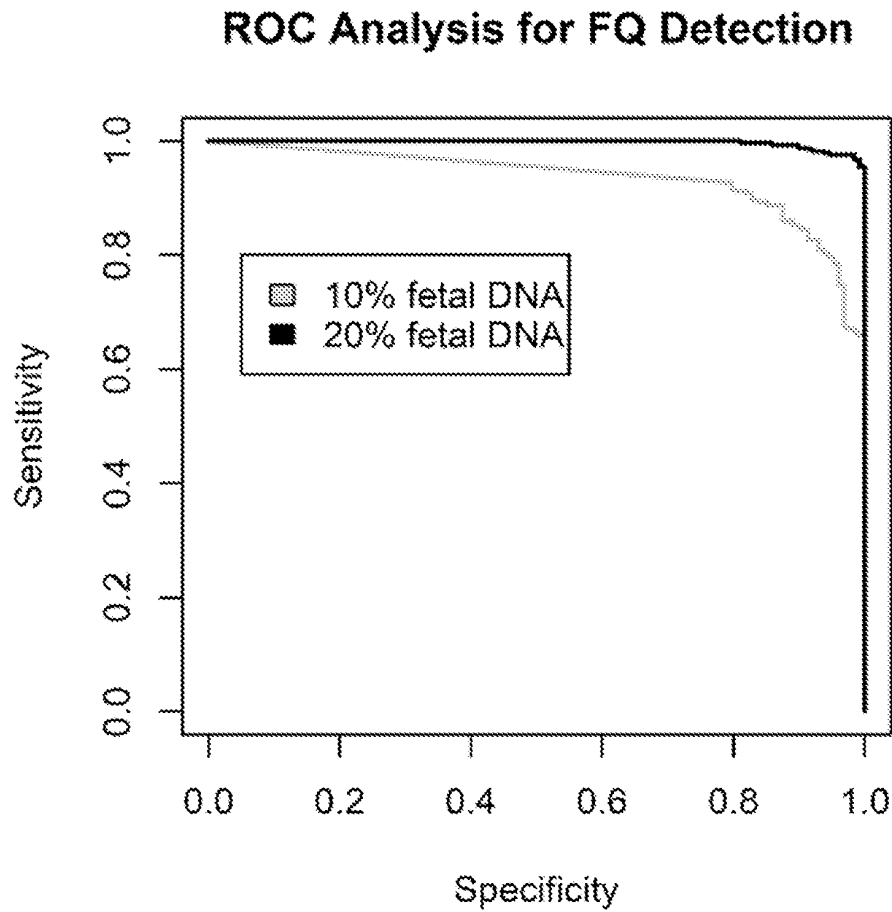


FIGURE 7

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FIGURE 8A

Non-invasive prenatal sex test - AMG_(F/M) -

	10	20	30	40	50	60
AM-X.SEQ	ACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAGGTAATTTTCTCTTTAC					
	X::					
AM-Y.SEQ	ACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAGGTAATTTTCTCTTTAC					
	10	20	30	40	50	60
	70	80	90	100	110	120
AM-X.SEQ	TAATTTTGACCATTTGTTGCGTTAACAATGCCCTGGGCTCTGTAAAGAATAGTGTGTTGA					
	::::::::: :: ::::: :: :: :: :: :: ::::::::::::::::::::::::::: :: ::					
AM-Y.SEQ	TAATTTTGATCACTGTTGCAATTAGCAGTCCCCTGGGCTCTGTAAAGAATAGTGGGTGGA					
	70	80	90	100	110	120
	130	140	150	160	170	
AM-X.SEQ	TTCTTTATCCCAGAT-----GTTTCTCAAGTGGTCCCTGATTTTACAGTTCCTACCACCA					
	::::: :::::X :: ::::::::::::::::::::::::::: :::::::::::::::::::::: ::					
AM-Y.SEQ	TTCTTCATCCCAAATAAAGTGTTTCTCAAGTGGTCCCAATTTTACAGTTCCTACCATCA					
	130	140	150	160	170	180
	180	190	200	210		
AM-X.SEQ	GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT					
	::					
AM-Y.SEQ	GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT					
	190	200	210			

Primers for non-invasive prenatal sex testing using AMG as target:

PCR primers:

AMG-F: 5'-ACGTTGGATGCCCTGGGCTCTGTAAAGAAT-3'
 AMG-R: 5'-ACGTTGGATGAGGCTTGAGGCCAACCATCAG-3'

EXTEND primers:

AMG-E: 5'-TTCTTCATCCCAAATAAAGT-3'

Competitors:

AMG-X-S:

5'-CCCTGGGCTCTGTAAAGAATAGTGTGTTGATTCTTTATCCCAGAAgTTTCTCAAGTGGTCCTGATTTTACAGTTCCTACCACCA
 GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT-3'

AMG-X-AS:

5'-AGGCTTGAGGCCAACCATCAGAGCTTAAACTGGGAAGCTGGTGGTAGGAAGTGTAAATCAGGACCACTTGAGAAACtTCTGGGATAAAGA
 ATCAACACACTATTCTTTACAGAGCCAGGG-3'

AMG-Y-S:

5'-CCCTGGGCTCTGTAAAGAATAGTGGGTGGATTCTTTCATCCCAAATAAAGTcGTTTCTCAAGTGGTCCCAATTTTACAGTTCCTACCATCA
 GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT-3'

AMG-Y-AS:

5'- AGGCTTGAGGCCAACCATCAGAGCTTAAACTGGGAAGCTGATGGTAGGAAGTGTAAATTTGGGACCACTTGAGAAACgACTTTATTTGGGATGA
 AGAATCCACCACTATTCTTTACAGAGCCAGGG

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FIGURE 8B

Non-invasive prenatal sex test - AMG-XY-5-i

	10	20	30	40	50	60
AM-X.SEQ	ACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAGGTAATTTTTCTCTTTAC					
	X::					
AM-Y.SEQ	ACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAGGTAATTTTTCTCTTTAC					
	10	20	30	40	50	60
	70	80	90	100	110	120
AM-X.SEQ	TAATTTTGACCATTGTTTGC GTTAACAATGCCCTGGGCTCTGTAAAGAATAGTGTTGA					
	::::::::: :: ::::: :: : : : ::::::::::::::::::::::::::::::::::: :: ::					
AM-Y.SEQ	TAATTTTGATCACTGTTTGCATTAGCAGTCCCTGGGCTCTGTAAAGAATAGTGGGTGGA					
	70	80	90	100	110	120
	130	140	150	160	170	
AM-X.SEQ	TTCTTTATCCCAGAT-----GTTTCTCAAGTGGTCCTGATTTTACAGTTCCTACCACCA					
	::::: :::::X :: ::::::::::::::: ::::::::::::::::::::::: ::					
AM-Y.SEQ	TTCTTCATCCCAAATAAAGTGGTTTCTCAAGTGGTCCCAATTTTACAGTTCCTACCATCA					
	130	140	150	160	170	180
	180	190	200	210		
AM-X.SEQ	GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT					
	::					
AM-Y.SEQ	GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT					
	190	200	210			

Primers for non-invasive prenatal sex testing using AMG as target:

PCR primers:

AMG-XY-5-i-F: 5'-ACGTTGGATGTATCAACTTCAGCTATGAGG-3'

AMG-XY-5-i-R: 5'-ACGTTGGATGCACTATTCTTTACAGAGC-3'

EXTEND primers:

AMG-XY-5-i-E: 5'-CTTTACAGAGCCCAGGG-3'

Competitors:

AMG-XY-5-i-S:

5'-TATCAACTTCAGCTATGAGGTAATTTTTCTCTTTACTAATTTTGAYCAYTGGTTGCRTTARCARTaCCCTGGGCTCTGTAAAGAATAGTG-3'

AMG-XY-5-i-AS:

5'-CACTATTCTTTACAGAGCCCAGGGtARTGRtAARGCAAACAYTGYTCAAAATTAGTAAAGAGAAAAATTACCTCATAGCTGAAGTTGATA-3'

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FIGURE 8C

Non-invasive prenatal sex test

```

              10      20      30      40      50      60
AM-X.SEQ      ACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAGGTAATTTTCTCTTTAC
X::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AM-Y.SEQ      ACCTCATCCTGGGCACCCTGGTTATATCAACTTCAGCTATGAGGTAATTTTCTCTTTAC
              10      20      30      40      50      60

              70      80      90      100     110     120
AM-X.SEQ      TAATTTTGACCATTTGTTGCGTTAACAATGCCCTGGGCTCTGTAAAGAATAGTGTTGA
:::::::::::: :: :::::: :: :: :: :: :::::::::::::::::::::::::::::: :: ::
AM-Y.SEQ      TAATTTTGATCACTGTTTGCATTAGCAGTCCCCTGGGCTCTGTAAAGAATAGTGGGTGGA
              70      80      90      100     110     120

              130     140     150     160     170
AM-X.SEQ      TTCTTTATCCCAGAT-----GTTTCTCAAGTGGTCCTGATTTTACAGTTCCTACCACCA
::::: :::::X :: :::::::::::::::::::::: :::::::::::::::::::::: ::
AM-Y.SEQ      TTCTTCATCCCAAATAAAAGTGGTTTCTCAAGTGGTCCCAATTTTACAGTTCCTACCATCA
              130     140     150     160     170     180

              180     190     200     210
AM-X.SEQ      GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT
::::::::::::::::::::::::::::::::::::::::::::::::::::::::::::
AM-Y.SEQ      GCTTCCCAGTTTAAGCTCTGATGGTTGGCCTCAAGCCT
              190     200     210

```

New primers for non-invasive prenatal sex testing using AMG as target:

PCR primers:

AMG-F: 5'-CCCTGGGCTCTGTAAAGAAT-3'

AMG-R: 5'-GAGCTTAACTGGGAAGCTG-3'

EXTEND primers:

AMG-Y: 5'-TTCTTCATCCCAAATAAAAGT-3'AMG-CON: 5'-CCCTGGGCTCTGTAAAGAATAGT-3'

EXTEND products:

Y chromosome: TTCTTCATCCCAAATAAAAGT**G**Template positive: CCCTGGGCTCTGTAAAGAATAGT**G**

RESULTS TABLE

Sequence Name	Primer Sequence	No. of Nucleotides	Mass
AMG-Y primer	TTCTTCATCCCAAATAAAAGT	20	6011
Ychromosome positive	TTCTTCATCCCAAATAAAAGTg	21	6340.2
AMG-CON primer	CTGGGCTCTGTAAAGAATAGT	21	6457.2
template positive	CTGGGCTCTGTAAAGAATAGTg	22	6786.4
SRY primer	caggacagcagtagagca	18	5550.6
SRY extension product	caggacagcagtagagcag	19	5879.8

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FIGURE 9

Non-invasive prenatal Albumin test

```

Query 1   GCTCAGTATCTTCAGCAGTGTCCATTGAAGATCAATGTAAAATTAGTGAATGAAGTAACT 60
          |||
Sbjct 193 GCTCAGTATCTTCAGCAGTGTCCATTGAAGATCATGTAAAATTAGTGAATGAAGTAACT 252

Query 61   GAATTTGC 68
          |||
Sbjct 253 GAATTTGC 260

```

ALB Assay:

PCR-F: 5'-ACGTTGGATGCAGTATCTTCAGCAGTGTCC-3'

PCR-R: 5'-ACGTTGGATGGCAAATTCAGTTACTTCATTC-3'

Extend: 5'-CAGTGTCCATTGAAGATC-3'

Competitor-S:

5'-CAGTATCTTCAGCAGTGTCCATTGAAGATCtTGTAATAATTAGTGAATGAAGTAACTGAATTTGC-3'

Competitor-AS:

5'-GCAAATTCAGTTACTTCATTCACCTAATTTTACAaGATCTTCAAATGGACACTGCTGAAGATACTG-3'

INTERNATIONAL SEARCH REPORT

International application No.

PCT/US 08/58317

A. CLASSIFICATION OF SUBJECT MATTER

IPC(8) - C12Q 1/68; C12P 19/34 (2008.04)

USPC - 435/6; 435/91.1

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC(8): C12Q 1/68; C12P 19/34 (2008.04)

USPC: 435/6, 91.1; 506/16

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

WEST (PGPB,USPT,EPAB,JPAB); restriction, endonuclease, polymorphic, loci, allele, target, paternal, maternal, pregnancy, non-thermostable, HpyCH4V; esp@cenet: sequenom, ehrlich
Google Scholar: HpyCH4V

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
Y	US 2007/0059707 A1 (CANTOR et al.) 15 March 2007 (15.03.2007), para [0010], [0012], [0013], [0016], [0020], [0029], [0031], [0037], [0039], [0066], [0068], [0070].	1-8
Y	US 2002/0064791 A1 (WHITAKER et al.) 30 May 2002 (30.05.2002), abstract; pg 3; para [0021]; claim 6.	1-8
Y	US 2004/0229224 A1 (FRAZER et al.) 18 November 2004 (18.11.2004), abstract; para [0073].	7
Y	NCBI: Single Nucleotide Polymorphisms. 25-05-2006, [online], [retrieved on 2008-06-03], Retrieved from the National Center for Biotechnology Information (at NIH) database using internet: <URL: http://www.ncbi.nlm.nih.gov/SNP/snp_ref.cgi?type=rs&rs=4329520>	8

☐ Further documents are listed in the continuation of Box C. ☐

* Special categories of cited documents:	"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A" document defining the general state of the art which is not considered to be of particular relevance	"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E" earlier application or patent but published on or after the international filing date	"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
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Date of the actual completion of the international search 4 June 2008 (04.06.2008)	Date of mailing of the international search report 30 JUN 2008
Name and mailing address of the ISA/US Mail Stop PCT, Attn: ISA/US, Commissioner for Patents P.O. Box 1450, Alexandria, Virginia 22313-1450 Facsimile No. 571-273-3201	Authorized officer: Lee W. Young PCT Helpdesk: 571-272-4300 PCT OSP: 571-272-7774

Form PCT/ISA/210 (second sheet) (April 2007)

Exhibit 19

(12) INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(19) World Intellectual Property Organization
International Bureau(43) International Publication Date
29 January 2009 (29.01.2009)

PCT

(10) International Publication Number
WO 2009/013492 A1

(51) International Patent Classification:

G06F 19/00 (2006.01) C12Q 1/68 (2006.01)

(21) International Application Number:

PCT/GB2008/002524

(22) International Filing Date: 23 July 2008 (23.07.2008)

(25) Filing Language: English

(26) Publication Language: English

(30) Priority Data:

60/951,438 23 July 2007 (23.07.2007) US

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(81) Designated States (unless otherwise indicated, for every kind of national protection available): AE, AG, AL, AM, AO, AT, AU, AZ, BA, BB, BG, BH, BR, BW, BY, BZ, CA, CH, CN, CO, CR, CU, CZ, DE, DK, DM, DO, DZ, EC, EE, EG, ES, FI, GB, GD, GE, GH, GM, GT, HN, HR, HU, ID, IL, IN, IS, JP, KE, KG, KM, KN, KP, KR, KZ, LA, LC, LK, LR, LS, LT, LU, LY, MA, MD, ME, MG, MK, MN, MW, MX, MY, MZ, NA, NG, NI, NO, NZ, OM, PG, PH, PL, PT,

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(54) Title: DETERMINING A NUCLEIC ACID SEQUENCE IMBALANCE

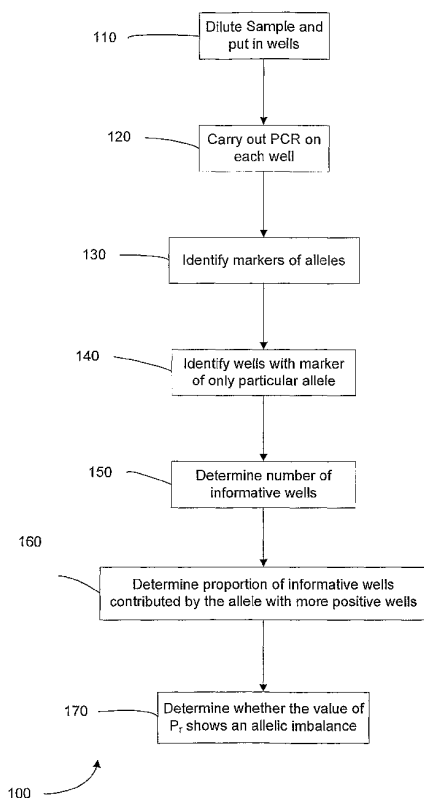


FIG. 1

(57) Abstract: Methods, systems, and apparatus are provided for determining whether a nucleic acid sequence imbalance exists within a biological sample. One or more cutoff values for determining an imbalance of, for example, the ratio of the two sequences (or sets of sequences) are chosen. The cutoff value may be determined based at least in part on the percentage of fetal DNA in a sample, such as maternal plasma, containing a background of maternal nucleic acid sequences. The cutoff value may also be determined based on an average concentration of a sequence per reaction. In one aspect, the cutoff value is determined from a proportion of informative wells that are estimated to contain a particular nucleic acid sequence, where the proportion is determined based on the above-mentioned percentage and/or average concentration. The cutoff value may be determined using many different types of methods, such as sequential probability ratio testing (SPRT).

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RO, RS, RU, SC, SD, SE, SG, SK, SL, SM, ST, SV, SY, TJ,
TM, TN, TR, TT, TZ, UA, UG, US, UZ, VC, VN, ZA, ZM,
ZW.

European (AT, BE, BG, CH, CY, CZ, DE, DK, EE, ES, FI,
FR, GB, GR, HR, HU, IE, IS, IT, LT, LU, LV, MC, MT, NL,
NO, PL, PT, RO, SE, SI, SK, TR), OAPI (BF, BJ, CF, CG,
CI, CM, GA, GN, GQ, GW, ML, MR, NE, SN, TD, TG).

(84) Designated States (*unless otherwise indicated, for every
kind of regional protection available*): ARIPO (BW, GH,
GM, KE, LS, MW, MZ, NA, SD, SL, SZ, TZ, UG, ZM,
ZW), Eurasian (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM),

Published:

— *with international search report*

DETERMINING A NUCLEIC ACID SEQUENCE IMBALANCE

CLAIM OF PRIORITY

[0001] The present application claims priority from and is a non-provisional application of U.S. Provisional Application No. 60/951438, entitled "DETERMINING A NUCLEIC ACID SEQUENCE IMBALANCE" filed July 23, 2007 (Attorney Docket No. 016285-005200US), the entire contents of which are herein incorporated by reference for all purposes.

CROSS-REFERENCES TO RELATED APPLICATIONS

[0002] The present application is also related to concurrently filed non-provisional application entitled "DIAGNOSING FETAL CHROMOSOMAL ANEUPLOIDY USING GENOMIC SEQUENCING," (Attorney Docket No. 016285-005220US), the entire contents of which are herein incorporated by reference for all purposes.

FIELD OF THE INVENTION

[0003] This invention generally relates to the diagnostic testing of genotypes and diseases by determining an imbalance between two different nucleic acid sequences, and more particularly to the identification of Down syndrome, other chromosomal aneuploidies, mutations and genotypes in a fetus via testing a sample of maternal blood. The invention also relates to the detection of cancer, the monitoring of transplantation, and the monitoring of infectious diseases.

BACKGROUND

[0004] Genetic diseases, cancers, and other conditions often result from or produce an imbalance in two corresponding chromosomes or alleles or other nucleic acid sequences. That is an amount of one sequence relative to another sequence is larger or smaller than normal. Usually, the normal ratio is an even 50/50 ratio. Down Syndrome (trisomy 21) is such a disease having an imbalance of an extra chromosome 21.

[0005] Conventional prenatal diagnostic methods of trisomy 21 involve the sampling of fetal materials by invasive procedures such as amniocentesis or chorionic villus sampling, which pose a finite risk of fetal loss. Non-invasive procedures, such as screening by ultrasonography and biochemical markers, have been used to risk-stratify pregnant women prior to definitive invasive diagnostic procedures. However, these screening methods typically measure epiphenomena that are associated with trisomy 21 instead of the core

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chromosomal abnormality, and thus have suboptimal diagnostic accuracy and other disadvantages, such as being highly influenced by gestational age.

[0006] The discovery of circulating cell-free fetal DNA in maternal plasma in 1997 offered new possibilities for noninvasive prenatal diagnosis (Lo, YMD and Chiu, RWK 2007 *Nat Rev Genet* 8, 71-77). While this method has been readily applied to the prenatal diagnosis of sex-linked (Costa, JM et al. 2002 *N Engl J Med* 346, 1502) and certain single gene disorders (Lo, YMD et al. 1998 *N Engl J Med* 339, 1734-1738), its application to the prenatal detection of fetal chromosomal aneuploidies has represented a considerable challenge (Lo, YMD and Chiu, RWK 2007, *supra*). First, fetal nucleic acids co-exist in maternal plasma with a high background of nucleic acids of maternal origin that can often interfere with the analysis (Lo, YMD et al. 1998 *Am J Hum Genet* 62, 768-775). Second, fetal nucleic acids circulate in maternal plasma predominantly in a cell-free form, making it difficult to derive dosage information of genes or chromosomes within the fetal genome.

[0007] Significant developments overcoming these challenges have recently been made (Benachi, A & Costa, JM 2007 *Lancet* 369, 440-442). One approach detects fetal-specific nucleic acids in the maternal plasma, thus overcoming the problem of maternal background interference (Lo, YMD and Chiu, RWK 2007, *supra*). Dosage of chromosome 21 was inferred from the ratios of polymorphic alleles in the placenta-derived DNA/RNA molecules. However, this method is less accurate when samples contain lower amount of the targeted gene and can only be applied to fetuses who are heterozygous for the targeted polymorphisms, which is only a subset of the population if one polymorphism is used.

[0008] Dhallan et al (Dhallan, R, *et al.* 2007, *supra* Dhallan, R, *et al.* 2007 *Lancet* 369, 474-481) described an alternative strategy of enriching the proportion of circulating fetal DNA by adding formaldehyde to maternal plasma. The proportion of chromosome 21 sequences contributed by the fetus in maternal plasma was determined by assessing the ratio of paternally-inherited fetal-specific alleles to non-fetal-specific alleles for single nucleotide polymorphisms (SNPs) on chromosome 21. SNP ratios were similarly computed for a reference chromosome. An imbalance of fetal chromosome 21 was then inferred by detecting a statistically significant difference between the SNP ratios for chromosome 21 and those of the reference chromosome, where significant is defined using a fixed p-value of ≤ 0.05 . To ensure high population coverage, more than 500 SNPs were targeted per chromosome. However, there have been controversies regarding the effectiveness of formaldehyde to

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enrich to a high proportion (Chung, GTY, et al. 2005 *Clin Chem* 51, 655-658), and thus the reproducibility of the method needs to be further evaluated. Also, as each fetus and mother would be informative for a different number of SNPs for each chromosome, the power of the statistical test for SNP ratio comparison would be variable from case to case (Lo, YMD & Chiu, RWK. 2007 *Lancet* 369, 1997). Furthermore, since these approaches depend on the detection of genetic polymorphisms, they are limited to fetuses heterozygous for these polymorphisms.

[0009] Using polymerase chain reaction (PCR) and DNA quantification of a chromosome 21 locus and a reference locus in amniocyte cultures obtained from trisomy 21 and euploid fetuses, Zimmermann et al (2002 *Clin Chem* 48, 362-363) were able to distinguish the two groups of fetuses based on the 1.5-fold increase in chromosome 21 DNA sequences in the former. Since a 2-fold difference in DNA template concentration constitutes a difference of only one threshold cycle (Ct), the discrimination of a 1.5-fold difference has been the limit of conventional real-time PCR. To achieve finer degrees of quantitative discrimination, alternative strategies are needed. Accordingly, some embodiments of the present invention use digital PCR (Vogelstein, B et al. 1999 *Proc Natl Acad Sci U S A* 96, 9236-9241) for this purpose.

[0010] Digital PCR has been developed for the detection of allelic ratio skewing in nucleic acid samples (Chang, HW et al. 2002 *J Natl Cancer Inst* 94, 1697-1703). Clinically, it has been shown to be useful for the detection of loss of heterozygosity (LOH) in tumor DNA samples (Zhou, W. et al. 2002 *Lancet* 359, 219-225). For the analysis of digital PCR results, sequential probability ratio testing (SPRT) has been adopted by previous studies to classify the experimental results as being suggestive of the presence of LOH in a sample or not (El Karoui et al. 2006 *Stat Med* 25, 3124-3133). In methods used in the previous studies, the cutoff value to determine LOH used a fixed reference ratio of the two alleles in the DNA of 2/3. As the amount, proportion, and concentration of fetal nucleic acids in maternal plasma are variable, these methods are not suitable for detecting trisomy 21 using fetal nucleic acids in a background of maternal nucleic acids in maternal plasma.

[0011] It is desirable to have a noninvasive test for fetal trisomy 21 (and other imbalances) detection based on circulating fetal nucleic acid analysis, especially one that is independent of the use of genetic polymorphisms and/or of fetal-specific markers. It is also desirable to have accurate determination of cutoff values and counting of sequences, which can reduce the

WHAT IS CLAIMED IS:

1 1. A method for determining whether a nucleic acid sequence imbalance
2 exists within a biological sample, the method comprising:
3 receiving data from a plurality of reactions, wherein the data includes:
4 (1) a first set of quantitative data indicating a first amount of a
5 clinically relevant nucleic acid sequence; and
6 (2) a second set of quantitative data indicating a second amount of a
7 background nucleic acid sequence different from the clinically relevant nucleic acid
8 sequence;
9 determining a parameter from the two data sets;
10 deriving a first cutoff value from an average concentration of a reference
11 nucleic acid sequence in each of the plurality of reactions, wherein the reference nucleic acid
12 sequence is either the clinically relevant nucleic acid sequence or the background nucleic acid
13 sequence;
14 comparing the parameter to the first cutoff value; and
15 based on the comparison, determining a classification of whether a nucleic
16 acid sequence imbalance exists.

1 2. The method of claim 1 wherein the first set of data are obtained from
2 one or more first markers that each detect a presence of a part of the clinically relevant
3 nucleic acid sequence in a reaction, and wherein the second set of data are obtained from one
4 or more second markers that each detect a presence of a part of the background nucleic acid
5 sequence in a reaction.

1 3. The method of claim 1, further comprising:
2 determining the average concentration of the reference nucleic acid sequence
3 in each of the plurality of reaction using an inverse of a probability distribution having an
4 input of a value derived from the data for the reference nucleic acid sequence.

1 4. The method of claim 1 wherein the clinically relevant nucleic acid
2 sequence is from chromosome 21 and the background nucleic acid sequence is from a
3 chromosome other than chromosome 21.

1 5. The method of claim 1 wherein the clinically relevant nucleic acid
2 sequence is from chromosome 18 or 13 and the background nucleic acid sequence is from a
3 chromosome other than chromosome 18 or 13 respectively.

1 6. The method of claim 1 wherein the clinically relevant nucleic acid
2 sequence is an allele of a genetic polymorphism, and the background nucleic acid sequence is
3 another allele of the genetic polymorphism.

1 7. The method of claim 1 wherein the clinically relevant nucleic acid
2 sequence is a mutated copy of the cystic fibrosis transmembrane conductance regulator
3 (CFTR) gene, the beta-globin gene or the alpha-globin gene, and the background nucleic acid
4 sequence is from the wildtype copy of the corresponding gene.

1 8. The method of claim 1 wherein the biological sample is plasma or
2 serum from a pregnant woman.

1 9. The method of claim 1 wherein a reaction is an amplification reaction.

1 10. The method of claim 9 wherein a reaction is a part of a digital PCR
2 process.

1 11. The method of claim 1 wherein a reaction is a sequencing reaction.

1 12. The method of claim 1 wherein first portions of the clinically relevant
2 nucleic acid sequence and the background nucleic acid sequence are from a first individual
3 and second portions of the clinically relevant nucleic acid sequence and the background
4 nucleic acid sequence are from a second individual.

1 13. The method of claim 12 wherein the cutoff value is based on a
2 measurement of one of the first portions or a measurement of one of the second portions.

1 14. The method of claim 1, further comprising comparing the parameter to
2 a second cutoff value.

1 15. The method of claim 14 wherein the classifications include disease
2 state, non-disease state, and non-classifiable.

1 16. The method of claim 14 wherein the classifications include
2 homozygous, heterozygous, and non-classifiable.

1 17. The method of claim 14 wherein the second cutoff value is based on a
2 ratio of the first amount of the clinically relevant nucleic acid sequence relative to the second
3 amount of the background nucleic acid sequence in a non-diseased state.

1 18. The method of claim 1 wherein the parameter is a ratio of the first
2 amount of the clinically relevant nucleic acid sequence relative to the second amount of the
3 background nucleic acid sequence.

1 19. The method of claim 1 wherein calculating the first cutoff value
2 includes using at least one of: sequential probability ratio testing, false discovery rates,
3 confidence intervals, and receiver operating characteristic curves.

1 20. The method of claim 1 wherein deriving the first cutoff value includes:
2 determining a proportion P1 of informative reactions containing an
3 overrepresented nucleic acid sequence, which is either the reference or non-reference nucleic
4 acid sequence; and
5 calculating the first cutoff value from the first proportion P1.

1 21. The method of claim 20 wherein determining the proportion P1
2 comprises:
3 determining a first probability of a reaction containing at least one of the
4 overrepresented nucleic acid sequence;
5 calculating a second probability that a reaction is informative; and
6 calculating the proportion P1 using the first probability and the second
7 probability.

1 22. The method of claim 21 wherein the first probability is determined by
2 multiplying the average concentration of the reference nucleic acid sequence by an expected
3 ratio relative to the non-reference nucleic acid sequence.

1 23. The method of claim 21 wherein the first probability is determined
2 using the Poisson distribution having the average concentration of the overrepresented
3 nucleic acid sequence in each of the plurality of reactions as an input.

1 24. The method of claim 21, further comprising:
2 determining a third probability of a reaction containing at least one of the
3 underrepresented nucleic acid sequence, wherein calculating the second probability that the
4 reaction is informative includes assuming that first probability and the second probability are
5 independent.

1 25. A method for determining whether a nucleic acid sequence imbalance
2 exists within a biological sample, the method comprising:
3 receiving data from a plurality of reactions, wherein the data includes:
4 (1) a first set of quantitative data indicating a first amount of a
5 clinically relevant nucleic acid sequence; and
6 (2) a second set of quantitative data indicating a second amount of a
7 background nucleic acid sequence different from the clinically relevant nucleic acid
8 sequence, wherein the clinically relevant nucleic acid sequence and the background nucleic
9 acid sequence come from a first type of cells and from one or more second types of cells;
10 determining a parameter from the two data sets;
11 deriving a first cutoff value from a first percentage resulting from a
12 measurement of an amount of a nucleic acid sequence from the first type of cells in the
13 biological sample;
14 comparing the parameter to the first cutoff value; and
15 based on the comparison, determining a classification of whether a nucleic
16 acid sequence imbalance exists.

1 26. The method of claim 25 wherein the first type of cells is from a first
2 organism and the second types of cells are from a second organism.

1 27. The method of claim 25 wherein deriving the first cutoff value
2 includes:
3 determining a first average concentration of a reference nucleic acid sequence
4 per reaction, wherein the reference nucleic acid sequence is the clinically relevant nucleic
5 acid sequence or the background nucleic acid sequence that is underrepresented; and
6 multiplying the first average concentration by a factor derived from the first
7 percentage to obtain a second average concentration of the nucleic acid sequence that is not
8 the reference nucleic acid sequence.

28. The method of claim 27, further comprising:

determining the average concentration of the reference nucleic acid sequence in each of the plurality of reactions using an inverse of a probability distribution having an input of a value derived from the data for the reference nucleic acid sequence.

29. The method of claim 28 wherein the probability distribution is the

Poisson distribution.

30. The method of claim 25 wherein the percentage is measured by

determining an amount of a fetal-specific marker using quantitative real-time PCR, digital PCR, semiquantitative competitive PCR, real-competitive PCR, or mass spectrometry.

31. A computer program product comprising a computer readable medium

encoded with a plurality of instructions for controlling a computing system to perform an operation for determining whether a nucleic acid sequence imbalance exists within a biological sample, the operation comprising the steps of:

receiving data from a plurality of reactions, wherein the data includes:

(1) a first set of quantitative data indicating a first amount of a clinically relevant nucleic acid sequence; and

(2) a second set of quantitative data indicating a second amount of a background nucleic acid sequence different from the clinically relevant nucleic acid sequence;

determining a parameter from the two data sets;

deriving a first cutoff value from an average concentration of a reference nucleic acid sequence in each of the plurality of reactions, wherein the reference nucleic acid sequence is either the clinically relevant nucleic acid sequence or the background nucleic acid sequence;

comparing the parameter to the first cutoff value; and

based on the comparison, determining a classification of whether a nucleic acid sequence imbalance exists.

Exhibit 20

APPENDIX A

DECLARATION OF PROFESSOR JOHN QUACKENBUSH, PH.D.

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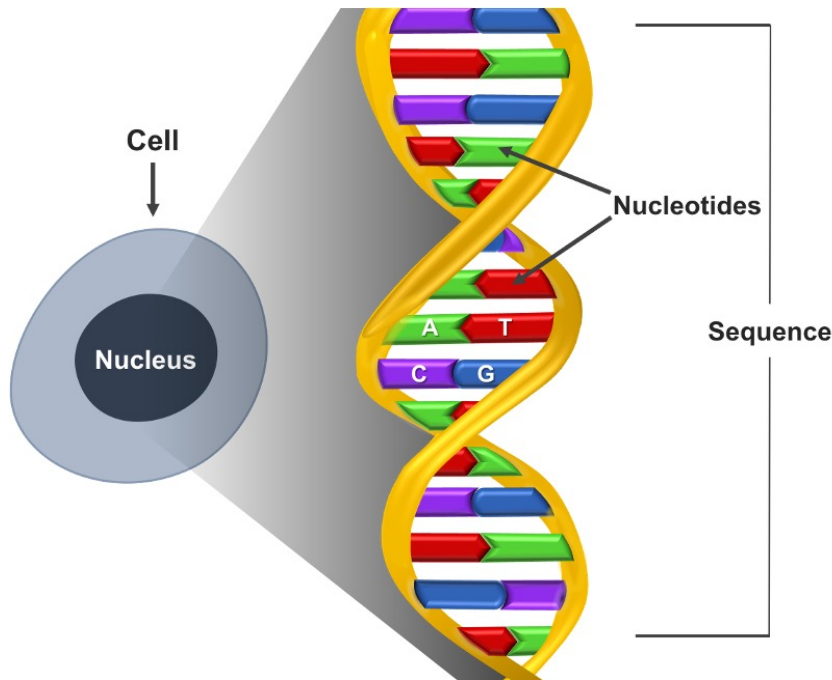
VI. BACKGROUND ON THE TECHNOLOGY

A. Nucleic Acids And Genes

39. Nucleic acids are biomolecules that are present in all living things. They store and encode the information living cells need to grow and function. Although there are many different subtypes of nucleic acids, naturally occurring, biologically active nucleic acids fall into two main classes: deoxyribonucleic acid (“DNA”) and ribonucleic acid (“RNA”).

40. DNA naturally exists as two long strands in a double-helical structure. Each strand is comprised of a sequence of “nucleotides” connected at regular intervals along the strand. On each nucleotide is one of several different types of “bases.” There are four natural types of bases that occur in DNA: adenine (“A”), cytosine (“C”), guanine (“G”) and thymine (“T”), which pair together complementarily (A to T, and C to G) to hold the two strands of DNA together. Each base, with its corresponding piece of the backbone of the DNA strand, is referred to as a “nucleotide.”

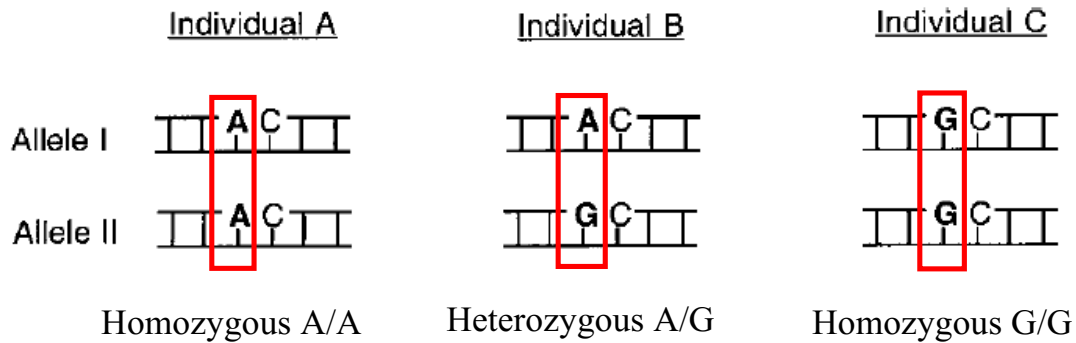
41. An individual’s DNA is organized into chromosomes, which reside in the nucleus of eukaryotic cells. This, and the general structure of DNA, is depicted in the figure below:



B. Genes, Polymorphisms, Homozygosity, And Heterozygosity

42. It is the unique sequence of bases in DNA that creates a code, or blueprint, for the production of other molecules such as proteins, which form cells' structures and make them function.

43. All individuals (apart from identical twins) have unique DNA sequences that genetically distinguish one person from another. The genetic constitution of an individual is called a "genotype." The differences in genetic sequences that give rise to everyone's unique genotype are called "polymorphisms." A "single nucleotide polymorphism," or "SNP," occurs where the nucleotide at a specific location may differ between different individuals. SNPs occur at about one



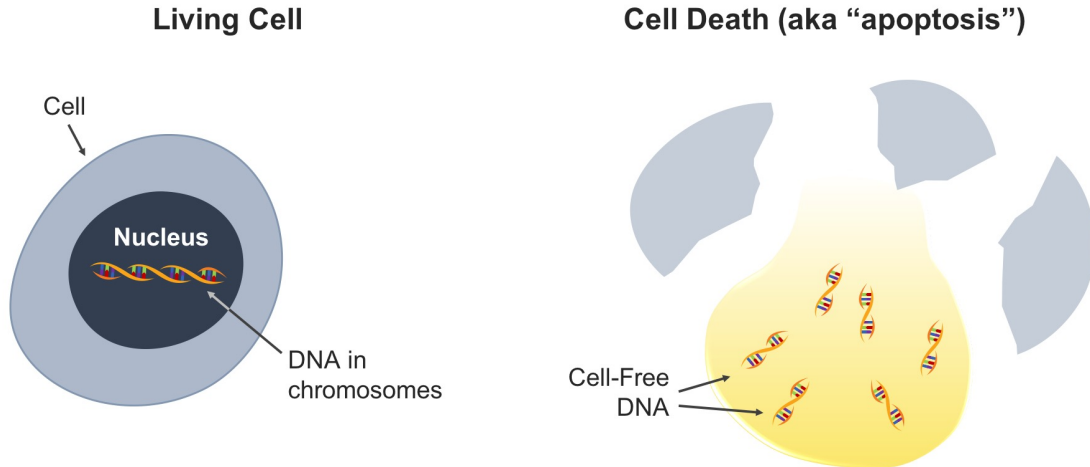
45. There is on average one SNP in every ~1,000 nucleotides in humans, and two individuals on average vary in approximately ~3,000,000 SNPs. B0001-B0024 ('652 Patent) at B0016, 13:41-44; B0481-B0580, (Vogel 2009) at B0574. Given the diversity in SNPs among individuals, scientists have exploited SNPs for many different purposes, including identifying people in forensic analyses, or determining patterns of SNPs associated with specific populations or disease states.

46. In addition to SNPs, there are other types of genetic variations. For example, "VNTRs," or variable numbers of tandem repeats, are short sequence repeats that are polymorphic due to variable numbers of short sequence repeat units as between individuals. While "dinucleotide repeats" are most common VNTRs can also be tri-, tetra-, or penta-repeats (often called microsatellites). VNTRs with longer repeat units of 15-500 nucleotides are called "minisatellites." "Insertion" and "deletion" polymorphisms are due to the presence of additional sequences or

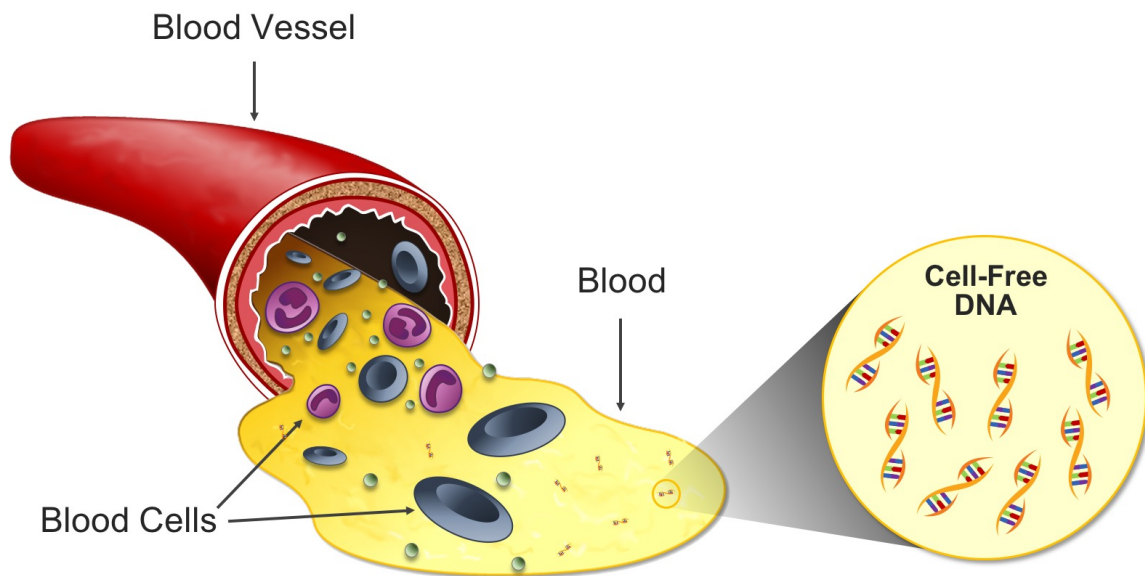
absence of normally present sequences, respectively. Regions of DNA that have many of the polymorphisms described above are commonly known as “hypervariable regions.”

C. Cell-Free Nucleic Acids

47. As discussed above, human DNA is most commonly found in the nuclei of cells in chromosomes. But DNA also can naturally exist outside of the cell. What scientists typically refer to as “cell-free” nucleic acids (and in the case of DNA, cell-free DNA or “cfDNA”) are small fragments of nucleic acids that are released from cells into the bloodstream. They circulate freely in the blood and other bodily fluids. These cell-free nucleic acids are usually produced when cells die, in a process called “apoptosis.” In apoptosis, cells undergo various biochemical processes that cause the nucleic acids, including DNA of the cells’ chromosomes, to be cleaved and fragmented before being released into the circulation. They are eventually cleared out of the body with other bodily waste products. The process of release of cfDNA from apoptotic cells is illustrated below:



48. Because cells are constantly dying and being replaced throughout the body, cell-free nucleic acids, including cfDNA, are constantly released into and circulating through the bloodstream or other body fluids, such as urine, before being cleared out of the body, as depicted below:



D. Cell-Free Nucleic Acids Having Sequences Different From A Patient's Genotype Occur Naturally In An Individual's Circulation

49. In certain diseases and conditions, nucleic acids including cfDNA that are foreign or abnormal to a person are present in the person's body. This occurs in a variety of contexts where a source of foreign DNA, or cells with different genotypes, reside in the host. Some of these are discussed below.

1. Cell-Free Nucleic Acids In Organ Transplant

50. For example, a transplanted organ can be a source of a genotype that is different from that of the transplant recipient. If an organ from a donor is transplanted into the body of a recipient, then the donor's DNA, with its unique genotype, is also transplanted into the body of the recipient. Because a transplanted organ's cells naturally die at a certain rate, just like all of the cells in the rest of a recipient's body, cell-free nucleic acids from the donated transplant organ will be released into, and circulate in, the recipient's blood along with the cell-free nucleic acids released from the cells in all of the rest of the recipient's body. This gives rise to two different genotypes being present in the same individual recipient's blood.

51. While many transplanted organs remain healthy, sometimes the body of the transplant recipient will reject the transplanted organ, or the transplanted organ will fail to survive for some other reason in the transplant recipient's body. Where

rejection occurs, the recipient's immune system attacks the transplanted organ and kills its cells, causing the cells to die more rapidly than they normally would. In transplant failure for other reasons, the cells of the transplanted organ die more rapidly than normal for a variety of reasons. Whether the transplant is rejected or otherwise fails, the transplanted organ's cells release more cell-free nucleic acids into the recipient's circulation than would be expected from a healthy transplant. These elevated levels of cell-free nucleic acids originating from the donor organ is the natural result of biological processes occurring in the transplant recipient's body. As of the priority date, it was well known that donor-derived DNA is present in a transplant recipient. *See, e.g.*, B0001-B0024 ('652 Patent) at B0013, 7:53-8:22; B0080-B0081 (Lo 1998) at B0080.

2. Cell-Free Nucleic Acids In Pregnancy

52. Another example occurs in the case of pregnancy, as the fetus growing in the body of its mother has a genotype that is different from its mother. As of the priority date, it was well-known that fetal DNA is naturally found as cfDNA in the pregnant mother's bloodstream, as the result of release from fetal cells that die while the fetus is growing in the mother's body. *See, e.g.*, B0001-B0024 ('652 Patent) at B0013, 7:19-23. This also gives rise to two different genotypes being present in an individual pregnant woman's blood.

53. Cell-free nucleic acids released from fetal cells into the mother's blood will reflect the fetus's genotype. Some fetuses have certain conditions such as disease, or an extra or missing chromosome ("aneuploidy," and for example, Down Syndrome). These are conditions of the fetus that are detectable while the fetus is in the womb through the fetal cell-free nucleic acids being released into the mother's bloodstream. For example, if the fetus is aneuploid, the fetal cfDNA from the extra or missing chromosome will be present in the mother's blood in greater or lesser quantity than would be expected if the fetus were not aneuploid. This observable difference, like the observable difference in a transplant recipient, is the natural result of biological processes occurring in the pregnant woman's body.

3. Cell-Free Nucleic Acids In Cancer

54. Yet another example occurs in the case of cancer, where a tumor with a unique genotype as a result of mutations grows in the body of a cancer patient. The sequences of the DNA in the tumor cells differ from those of the cells in the rest of the cancer patient, which do not have the mutations unique to the tumor. Because these tumor cells die and release their cell-free nucleic acids into the cancer patient, where they circulate together with the cell-free nucleic acids released from the non-cancerous cells in the body, the tumor acts as a source of an additional, different genotype present in the cancer patient. As of the priority date, it was well known that

the DNA sequences of tumor cells differ from the DNA of a cancer patient. *See, e.g.*, B0001-B0024 ('652 Patent) at B0012, 6:67-7:5.

55. Cancer cell-free nucleic acids can serve as a source for monitoring cancer as well. The cancerous tumor cells have genetic mutations not present in the patient's normal cells, and as the tumor cells die (either because the patient's immune system attacks and kills them or they are starved for resources due to the expanding tumor mass) they release cell-free nucleic acids into the cancer patient's circulation. As a result, these genetically unique tumor cells release cell-free nucleic acids with genomes that are not normally present in the cancer patient's circulation. This phenomenon becomes more pronounced as tumors grow, and again, is the natural result of biological processes occurring in the cancer patient's body.

4. Cell-Free Nucleic Acids In Infectious Disease

56. In the case of infectious disease, bacteria or viruses invading a person's body have genotypes that are different than that of the person they are infecting. The bacteria or viruses are destroyed by the person's immune system, causing the release of bacterial or viral cell-free nucleic acids into the person's blood. Those bacterial or viral cell-free nucleic acids will have sequences that differ from those of the infected person, such that they act as a source of additional, different genotypes present in the infected person's body. As of the priority date, this, too, was well-known and understood by POSAs. *See* section VII.C.2. below.

5. Scientists Have Long Used Cell-Free Nucleic Acids To Monitor Disease

57. In each of the above-referenced settings (transplant, pregnancy, cancer, and infectious disease), cells from the source of foreign DNA naturally die while inside of the patient and release their nucleic acids to circulate as cell-free nucleic acids in the patient's body. The resulting presence of cell-free nucleic acids with a different genotype than the patient's normal genotype is a natural result of biological processes that occur in the transplant recipient's, mother's, or cancer or infected patient's bodies, respectively. Scientists knew of these natural phenomena long before the November 6, 2009 priority date of the Patents. As described here in my declaration, and in the Patents themselves, scientists routinely applied methods for detecting and analyzing the pertinent genetic differences in samples from patients containing different genotypes in order to study conditions relating to them.

58. The Patents acknowledge that cell-free nucleic acids, including cfDNA, can be found circulating in an individual's bodily fluids, and that these circulating cell-free nucleic acids can be correlated to diseases involving cell death, or apoptosis. As the written description explains:

Circulating, or cell-free, DNA was first detected in human blood plasma in 1948. ... Since then, its connection to disease has been established in several areas. ... Studies reveal that much of the circulating nucleic acids in blood arise from necrotic or apoptotic cells ... and greatly elevated levels of nucleic acids from apoptosis is observed in diseases such as cancer. ...

B0001-B0024 ('652 Patent) at B0012, 6:57-67 (citations omitted); *see also* Section VII.C.2. below.

VII. THE PATENT CLAIMS ARE DIRECTED TO WELL-UNDERSTOOD, ROUTINE AND CONVENTIONAL TECHNIQUES APPLIED TO OBSERVING A NATURAL PHENOMENON

A. The Claims of the Patents Are Directed to a Natural Law

59. It is my opinion that the claims of the Patents are directed to a natural law—the presence of naturally occurring donor-derived cell-free nucleic acids in the bodily fluids of transplant recipients, and the correlation of those naturally occurring cell-free nucleic acids to transplant status or rejection.

60. Claim 1 of the '652 Patent recites a “method for detecting transplant rejection, graft dysfunction, or organ failure” by “diagnosing, predicting, or monitoring a transplant status or outcome of the subject who has received the transplant by determining a quantity of the donor cell-free nucleic acids based on the detection of the donor cell free nucleic acids and subject cell-free nucleic acids by the multiplexed sequencing, wherein an increase in the quantity of the donor cell-free nucleic acids over time is indicative of transplant rejection, graft dysfunction or organ failure.” Thus, claim 1 of the '652 Patent provides that *if* an increase in donor cell-free nucleic acid is detected in a bodily fluid of a transplant recipient over time, *then* organ transplant rejections can be diagnosed, predicted or monitored.

61. Claim 1 of the '497 Patent recites a “method of detecting donor-specific circulating cell-free nucleic acids in a solid organ transplant recipient” by “determining an amount of donor-specific circulating cell-free nucleic acids from the solid organ transplant in the biological sample by detecting a homozygous or a heterozygous SNP ... in at least one assay ... wherein the at least one assay detects the donor-specific circulating cell-free nucleic acids from the solid organ transplant.” Thus, claim 1 of the '497 Patent starts with donor-specific cell-free nucleic acids that are already present in a sample, and as an end result detects them.

62. Claim 1 of the '607 Patent recites a “method of quantifying kidney transplant-derived circulating cell-free deoxyribonucleic acids in a human kidney transplant recipient” by “quantifying an amount of said kidney transplant-derived circulating cell-free deoxyribonucleic acid in said plasma sample to obtain a quantified amount.” This claim, too, starts with a certain quantity of transplant-derived cfDNA already present in a sample, and as an end result observes that quantity.

63. This presence of naturally occurring cell-free nucleic acids in the bodily fluids of transplant recipients, and its correlation to transplant status or rejection, exists apart from any human intervention. Cell death is a natural consequence of a transplanted organ's biological response to being rejected or otherwise failing. The natural result of this biological response is an elevated level of donor-specific cell-

free nucleic acids in the transplant recipient's bodily fluids. As explained in the Patents' written description, this is a natural phenomenon that does not require any human intervention. *See, e.g.*, B0001-B0024 ('652 Patent) at B0013, 7:40-46 (stating that "as cell-free DNA or RNA often arises from apoptotic cells, the relative amount of donor-specific sequences in circulating nucleic acids should provide a predictive measure of on-coming organ failure in transplant patients for many types of solid organ transplantation including, but not limited to, heart, lung, liver, and kidney.").

64. The independent claims of the Patents are representative of each of the dependent claims—all of which are directed to a natural law. Each dependent claim relies on the method of the independent claim of each patent. In addition, all recite substantially similar methods and all are linked to observing the same natural phenomenon of donor-specific cell-free nucleic acids in a transplant recipient's circulation, or its correlation to transplant rejection.

65. Beyond the elements of the claims from which they depend, the dependent claims of the Patents recite:

- Using different types or numbers of polymorphisms ('652 Patent Claims 2 and 11; '497 Patent Claims 6, 17, and 25; '607 Patent Claims 2, 3, 4, and 5)
- Using cf-DNA ('652 Patent Claim 3; '497 Patent Claim 12)
- Using certain common modifications, error rates, or quality scores associated with multiplexed or high throughput sequencing ('652 Patent Claims 4 and 6; '497 Patent Claims 3, 4, 5, 19, 21, and 23 '607 Patent Claim 6)
- Using known PCR or amplification methods '497 Patent Claim 10)

- Using different sample types ('652 Patent Claim 12; '497 Patent Claims 2 and 27)
- Further genotyping prior to quantifying cell-free nucleic acids ('497 Patent Claim 15)
- Certain concentrations of cell-free nucleic acids in the sample ('497 Patent Claim 20; '607 Patent Claims 7, 8, 9, and 10)

66. By depending on and incorporating the phenomenon of the presence of naturally occurring donor-derived cell-free nucleic acids in the circulation of transplant recipients and the natural law correlating it to transplant rejection, without adding more, each of the dependent claims of the Patents are also directed to this natural law.

B. Each Recited Laboratory Technique Was Conventional

67. In my experience as a researcher in the genomics field in the 2009 timeframe, all of the techniques recited in the Patent claims were well understood, routine and conventional as of their November 6, 2009 filing date. Indeed, the Patents' written description states: "The practice of the present invention employs, unless otherwise indicated, conventional techniques of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, which are within the skill of the art." B0001-B0024 ('652 Patent) at B0012, 5:36-40. I have reviewed the written description, including the portions referenced herein, and I have not seen any disclosure in which the Patents "otherwise indicate[]" that any of the laboratory techniques recited in the claims, or that the particular way they are combined in the claims, is nonconventional. To the contrary,

the written description repeatedly describes all of the recited techniques, and the claimed combinations of them, as conventional and routinely practiced using commercially available products.

68. As summarized above in section V, a POSA would understand the methods of the Patents to recite four common categories of laboratory techniques that were well-established approaches to cell-free nucleic acid detection and quantification by November, 2009:

- **Obtaining / providing a biological sample** containing cell-free nucleic acids from a transplant recipient;⁹
- **Genotyping** the transplant donor and/or recipient **to establish profiles of genetic polymorphisms (or SNPs)**;¹⁰
- **Performing multiplex or high-throughput sequencing** of the cell-free nucleic acids to detect the genotyped polymorphisms (or SNPs);¹¹ and

⁹ For independent claims, see '652 Patent Claim 1(a) (“providing a sample comprising [cfDNA]”); '497 Patent Claim 1(c) (“obtaining a biological sample”); '607 Patent Claims 1(a) and (b) (“providing a plasma sample” and “extracting circulating [cfDNA]”). For dependent claims that recite similar sampling and transplant-related limitations, see '497 Patent Claims 2, 9, 12, and 27.

¹⁰ For independent claims, see '652 Patent Claim 1(b) (“obtaining a genotype ... to establish a polymorphism profile”); '497 Patent Claims 1(a) and (b) (“genotyping ... to obtain a SNP profile”); and '607 Patent Claim 1(c) (“performing a selective amplification of [SNPs] ... by [PCR]”) and Claim 1(f) (“using markers distinguishable between said [recipient and donor] [that] comprise [SNPs]”). For dependent claims that recite similar genotyping and polymorphism-related limitations, see '652 Patent Claims 2 and 11; '497 Patent Claims 6, 15, 17, and 25; and '607 Patent Claims 2-5 recite similar genotyping and polymorphism-related limitations.

¹¹ For independent claims, see '652 Patent Claim 1(c) (“multiplex sequencing of the [cfDNA] in the sample followed by analysis of the sequencing results using the

- **Quantifying the transplant (donor-derived) cell-free nucleic acids in the sample using the genetic differences in the sequences.**¹²

69. The Patent claims identify the recited steps only at a high level of generalization. The written description identifies many different conventional ways each of the laboratory techniques recited in those steps, and the combinations of them, can be performed. However, the claims do not identify any particular approach to performing the steps or describe any unconventional performance of the common techniques already in use in 2009 as described in the written description.

70. As I explain in section VII.B.3. below, there was what those of us in the field have characterized as an explosion of technology driven in part by rapid advances in the sensitivity, accuracy, and throughput of DNA sequencing and analysis methods that had become routine and conventional for cell-free nucleic acid

polymorphism profile”); ’497 Patent Claim 1(d) (“determining an amount of donor-specific [cfDNA] by ... high-throughput sequencing or [dPCR]”); ’607 Patent Claims 1(d-e) (“performing a high throughput sequencing reaction ... compris[ing] ... sequencing-by-synthesis ... [and] ... providing sequences from said high throughput sequencing reaction”). For dependent claims that recite similar sequencing-related limitations, see ’652 Patent Claims 4 and 6; and ’497 Patent Claims 3-5, and 10.

¹² For independent claims, see ’652 Patent Claim 1(d) (“determining a quantity of [transplant cfDNA] based on the detection of [donor and recipient cfDNA] by the multiplexed sequencing”); ’497 Patent Claim 1(d) (“determining an amount of [transplant cfDNA] ... by detecting a homozygous or a heterozygous SNP within the [transplant cfDNA]”); and ’607 Patent Claim 1(f) (“quantifying an amount of [transplant cfDNA] ... using markers distinguishable between ... recipient and ... donor”).

detection as recited in the Patent claims in the years leading up to November 2009.

I know this from my own professional experience at the time, and it is corroborated by the literature.

71. For example, during the relevant 2009 time period I was a scientific advisor for Helicos Biosciences, Inc. (“Helicos”), which was co-founded by named Patent inventor Dr. Stephen Quake. A 10-K Annual Report for fiscal year 2007 from Helicos explains the state of the industry at that time, and reinforces my opinion that the laboratory techniques recited in the claims of the Patents were well understood, routine and conventional by November 2009:

Since the development of genetic engineering techniques in the 1970s, the analysis of genetic material has become a mainstay of biological research. The first automated DNA sequencer was invented in 1986, based on technology developed by Frederick Sanger and his colleagues in 1975, which is commonly referred to as Sanger sequencing. Subsequent versions of commercial DNA sequencers have increased the speed of DNA sequencing by 3,000 fold, making possible the Human Genome Project. In 1996 the first commercial microarray was introduced and enabled a new era of RNA analysis by measuring gene expression across many genes in a single experiment. Subsequent versions of the commercial microarrays including DNA and RNA have significantly increased the amount of information per run and provided selected SNPs of the whole human genome on a single chip, enabled large scale genome-wide SNP association studies and have been commercialized for several diagnostic applications. Today, manufacturers of systems, supplies and reagents for performing genetic analysis, which includes DNA sequencing, genotyping, and gene expression analysis, serve a worldwide market of approximately \$5 billion, according to Strategic Directions International. Strategic Directions International estimates that DNA sequencing serves approximately 17% of this demand for genetic analysis.

B0581-B0693 (Helicos 10-K) at B0587. As named inventor Dr. Quake's company acknowledged in its SEC filings, by the time of the Helicos 10-K in 2007, systems and methods for DNA sequencing and genotyping had become well understood, routine and conventional, and served a worldwide market of approximately \$5 billion. In its 10-K, Helicos also acknowledged "**established** genomic analysis technologies," including sequencing, "next generation sequencing" (which as explained in section VII.B.3. below is a term that for pertinent purposes is used interchangeably with multiplex sequencing or high-throughput sequencing), and genotyping (B0581-B0693 Helicos 10-K) at B0589:

Comparison of established genomic analysis technologies				
Analysis	Description	Technology	Advantages	Disadvantages
Sequencing	Determination of the complete sequences of DNA or RNA molecules	Automated Sanger-based instruments	<ul style="list-style-type: none"> • Comprehensive sequence information • Industry standard technology 	<ul style="list-style-type: none"> • High cost • Low throughput • Complex sample preparation
Next Generation Sequencing	Determination of the complete sequences of DNA and RNA molecules	Ensemble-on-bead based technologies	<ul style="list-style-type: none"> • Comprehensive sequence information • High throughput/lower cost per sequence • Seen as "upgrade" to Sanger sequencers 	<ul style="list-style-type: none"> • Complex sample preparation • Limited scalability • High cost of sample preparation • Limited quantitation
Gene Expression Analysis	Detection and quantitation of RNA to determine gene expression levels	DNA arrays on chips or beads	<ul style="list-style-type: none"> • Can perform genome-wide analysis of expressed genes • Widely available 	<ul style="list-style-type: none"> • Low sensitivity • Relative quantitation • Limited sequence information • Limited to known genomic sequences • Biased based on templates
		RT-PCR	<ul style="list-style-type: none"> • Absolute quantitation • Highest sensitivity 	<ul style="list-style-type: none"> • Higher cost per gene than arrays • Labor intensive • Not scalable
Genotyping	Analysis of short specific sequences within genomic DNA to look for known variants	DNA arrays on chips or beads	<ul style="list-style-type: none"> • High throughput/low cost per genotype • Can be applied to large numbers of samples 	<ul style="list-style-type: none"> • Provides only limited genomic information • Only interrogates known sequence variants
		RT-PCR	<ul style="list-style-type: none"> • Higher sensitivity than arrays 	<ul style="list-style-type: none"> • Provides very limited genomic information • Higher cost per genotype than arrays • Biased based on templates

72. The Patents' written description itself describes the recited methods as conventional. I summarize portions of the Patents' written description identifying the conventional nature of the recited method steps in turn below.

1. Obtaining Or Providing a Biological Sample Containing Cell-Free Nucleic Acids From a Transplant Recipient

73. '652 Patent Claim element 1(a), '497 Patent Claim element 1(c), and '607 Patent Claim element 1(a) recite providing or obtaining a sample comprising cell-free nucleic acids from a transplant recipient. Techniques for providing or

obtaining such samples were routine long before the 2009 filing of the Patents, which the Patents' written description confirms.

74. Obtaining and studying biological samples of cell-free nucleic acids from an individual was routine and conventional by 2009. As the Patents' written description explains, circulating or cell-free DNA was already well characterized in the prior art. B0001-B0024 ('652 Patent) at B0012, 6:57-58 (“[c]irculating, or cell-free, DNA was first detected in human blood plasma in 1948.”).

75. Techniques for obtaining biological samples were routine, conventional and well-established by 2009. For example, venipuncture, or the science of making punctures in a patient's vein with a needle in order to draw blood from the vein, was well known in the prior art. *Id.* at B0014, 10:11-12 (emphasis added) (“To obtain a blood sample, ***any technique known in the art may be used***, e.g., a syringe or other vacuum suction device ...”).

76. The '607 Patent, at Claim element 1(b), further recites extracting cfDNA from the plasma sample. This also was routine by 2009, with numerous options for commercial kits available to carry it out.

77. The Patents do not purport to claim an improvement over or nonconventional application of the traditional laboratory techniques for obtaining or providing biological samples comprising cell-free nucleic acids. I also have seen no indication in the claims or written description of the Patents that demonstrates an

improvement over or nonconventional application of those conventional techniques or a new or unique way of carrying them out.

2. Genotyping The Transplant Donor And/Or Recipient To Establish Profiles Of Genetic Polymorphisms (Or SNPs), Including By Comprising Selectively Amplifying At Least 1,000 SNPs By PCR And Sequencing

78. The '652 and '497 Patent claims also recite “genotyping” to establish “profiles” of “SNPs” or “polymorphisms,” including homozygous and/or heterozygous variants. *See* '652 Patent claim element 1(b) and '497 Patent claim elements 1(a) and (b). The '607 Patent, at elements 1(c)-(e), claims a generalized and conventional method for genotyping, reciting “performing a selective amplification of target [DNA] sequences” that “amplifi[es] ... at least 1,000 [homozygous and/or heterozygous] [SNPs]” by [PCR]” (Claim 1(c)), followed by “high throughput sequencing” (Claim 1(d)), and then “providing sequences” of the “[SNPs]” (Claim 1(e)). These claim limitations describe what a POSA in 2009 would recognize as a common approach to genotyping, including based on targeted PCR amplification and sequencing.

79. A POSA in 2009 would recognize that the Patents claim these steps at a high level without purporting to describe any improvements to then-existing genotyping techniques. As the written description explains, numerous techniques were known as of the November 6, 2009 filing date—including numerous

commercially available options—for accomplishing the genotyping and SNP/polymorphism profiles recited in the claims.

80. The written description explains that:

Genotyping of the transplant donor and/or the transplant recipient *may be performed by any suitable method known in the art including those described herein such as sequencing, nucleic acid array or PCR*. ... In some embodiments, the marker profile is a polymorphic marker profile. Polymorphic marker profile may comprise one or more single nucleotide polymorphisms (SNP's)

B0001-B0024 ('652 Patent) at B0019, 20:31-44 (emphasis added); *see also id.* at B0014, 9:8-14 (“Detection, identification and/or quantitation of the donor-specific markers (e.g. polymorphic markers such as SNPs) can be performed using real-time PCR, chips (e.g., SNP chips), *high-throughput shotgun sequencing* of circulating nucleic acids (e.g. cell-free DNA), *as well as other methods known in the art including the methods described herein.*”) (emphasis added).

81. Moreover, literature from as early as 2003 confirms that as of that time, approximately 2.8 million SNPs had been identified and were available in public databases. *See, e.g.*, B0086-B0093, (HapMap Project) at B0089; *see also* B0094-B0155 (International Human Genome Sequencing Consortium 2001) at B0094, B0098-B0099, B0101, B0145, B0148; B0156-B0163 (Human Genome Project NIH FAQ) at B0159; B0164-B0183 (1000 Genome Project) at B0164. Thus, the written description acknowledges, and the literature confirms (as also discussed in more detail below), that routine techniques including sequencing, nucleic acid array and

PCR were available and used for genotyping up to thousands (or more) of SNPs by 2009.

(a) PCR, Including Selective Amplification, Was a Well-Understood, Routine and Conventional Method For Genotyping By 2009

82. PCR, including selective amplification, was a well-understood, routine and conventional method for genotyping by 2009. The written description describes PCR-based commercial products that were available at the time of filing to perform the claimed methods under the heading “Genotyping”:

For example, after genotyping a transplant donor and transplant recipient, *using existing genotyping platforms known in the art including the one described herein*, one could identify approximately 1.2 million total variations between a transplant donor and transplant recipient. Usable SNPs may comprise approximately 500,000 *heterozygous* donor SNPs and approximately 160,000 *homozygous* donor SNPs. *Companies* (such as Applied Biosystems, Inc.) *currently offer both standard and custom-designed TaqMan probe sets* for SNP genotyping that can in principle target any desired SNP position for a *PCR-based assay* (Livak, K. L., Marmaro, J., Todd, J. A., Nature Genetics, 9, 341-342 (1995); De La Vefa, F. M., Lazaruk, K. D., Rhodes, M. D., Wenz, M. H., Mutation Research, 573, 111-135 (2005)). With such a large pool of potential SNPs to choose from, a *usable subset of existing or custom probes* can be selected to serve as the probe set for any donor/recipient pair.”

B0001-B0024 (’652 Patent) at B0016, 13:51-67 (emphasis added). As noted by the dates of the references cited in this portion of the written description, these PCR-based tools for genotyping SNPs, including for genotyping up to hundreds of thousands of SNPs, were commercially available as far back as 1995.

83. By 2000, PCR had been universally adopted as a standard tool in applications of molecular biology, and it was already successful in the simultaneous detection of thousands of SNPs. *See e.g.*, B1291-B1300 (Germer 2000) at B1291. In fact, by 2009, PCR was also being widely used to measure SNPs in cell-free DNA. *See, e.g.*, B0191-B0212 (Mei 2005) at B0196; B0213-B0223 (Lichtenstein 2001) at B0214. PCR was a well-established, conventional and indispensable tool for genetic testing that was routinely used to target and amplify specific, pre-selected genes for further study. *See e.g.*, B0001-B0024 ('652 Patent) at B0016, 14:29-67.

84. By 2009, PCR also was commonly used to amplify nucleic acids, including to selectively amplify nucleic acids, in order to obtain sufficient amounts of them for “sequencing.” As discussed in more detail below, nucleic acid sequencing is the process of determining the nucleic acid sequence, or the order of nucleotide bases, in a nucleic acid. “High-throughput sequencing” as recited in '607 Patent Claim element 1(d) (and '497 Patent Claim element 1(d)) is a term commonly understood in the art of nucleic acid analysis to refer to sequencing technologies that sequence multiple DNA molecules in parallel. As discussed in section VII.B.3. below, this, too, had been widely accepted and had become a routine tool for nucleic acid analysis by 2009.

85. For example, by 2008, a product called RDT 1000 from the company RainDance Technologies combined “targeted sequencing” by PCR of “hundreds to

thousands of genomic loci.” B0224-B0225 (RainDance 2008) at B0224. As the manufacturer indicated, “[t]he RDT 1000 fits seamlessly into any targeted sequencing workflow,” and its “‘open’ design easily integrates with all next-generation DNA sequencing platforms.” *Id.*

86. Several other products for selective amplification were on the market by November 6, 2009, including from Agilent Technologies, Roche, and Febit. *See, e.g.*, B0226-B0243 (Volkerding 2009) at B0233. For example, in a Review article in 2009, Volkerding *et al.* described several “targeted genomic resequencing” products available at the time that combine selectively amplifying “polymorphisms” and other genetic regions of interest, and then sequencing them using multiplex or high-throughput sequencing instruments. *Id.* Volkerding *et al.* identified using “[c]apture probes [] immobilized on a solid surface,” as provided in the Roche NimbleGen product, Agilent products, and Febit products, or capture probes that are “used in solution,” as in other Agilent products. *Id.* These arrays could capture up to “350,000” sequences. *Id.* Using these products, the target DNA for selective amplification would be captured by probes on an array or in solution, then “the enriched DNA amplified by PCR before NGS [*e.g.*, high-throughput sequencing] library preparation.” *Id.*

87. I also have significant experience using these standard PCR techniques for genotyping prior to 2009. Starting in 1992, I ran a large program at the Salk

Institute using PCR to assay polymorphic genetic markers to produce a map of human chromosome 11, a project that required tens of thousands of multiplexed PCR reactions. Between 1994 and 1996, while at Stanford University, I was responsible for the design and conduct of hundreds of thousands of PCR reactions for mapping the entire human genome and as part of developing a strategy for sequencing regions of human chromosomes 4 and 21. After joining The Institute for Genomic Research in 1997, I was responsible for projects involving hundreds of thousands of PCR reactions for genome sequencing and microarray analysis. And after joining the faculty at the Dana-Farber Cancer Institute and Harvard School of Public Health, I led large-scale projects analyzing gene expression in cancer and establishing high-throughput genome sequencing that required PCR and other amplification reactions from diverse biological samples, including cell lines, human tissue samples, circulating tumor cells, and circulating cell-free DNA. In all of these projects, I used standard and routine PCR applications for genotyping.

(b) Arrays Were a Well-Understood, Routine and Conventional Method For Genotyping By 2009

88. Use of arrays was also a well-understood, routine and conventional method for genotyping by 2009. The written description states that genotyping SNPs could also be executed using arrays, including commercially available arrays manufactured by Illumina, Inc., ParAllele, and Affymetrix. B0001-B0024 ('652 Patent) at B0013, 8:55-60; B0018, 17:40-18:53.

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89. DNA microarrays were developed in the 1990s and essentially allow the detection of many unique fragments of DNA in parallel. DNA microarrays are based on the simple concept of printing specific sequences of DNA fragments onto different spots on a glass and detecting complementary sequences of DNA that bind to each spot. Using DNA fragments containing SNPs was a routine application of this technology. Indeed, Affymetrix developed the first commercial microarray system (the Affymetrix GeneChip®) in 1994, and in 1999, released an array called GeneChip HuSNP, consisting of a panel of 1,200 SNPs printed onto the array.

90. After the human genome was sequenced and the HapMap project was launched in 2002, there was intense interest in developing SNP arrays that could measure enough SNPs to genotype the entire genome of an individual on a single array. The NIH selected ParAllele and Illumina as two of the five US HapMap project participants responsible for genotyping. *See* B0244-B0250 (Ogren 2003) at B0248. In connection with this, ParAllele partnered with Affymetrix to develop a universal array system that could be used with any custom SNP. Illumina similarly adapted its GoldenGate® Assay to be used with a universal BeadChip to analyze large numbers of SNPs. The Illumina and ParAllele/Affymetrix arrays became standard SNP genotyping platforms by the time the HapMap project was launched in 2002.

91. Applied Biosystems TaqMan probe sets were first described in 1995 by Kenneth J. Livak and Jeffrey Marmaro. *See* B0184-B0185 (Livak 1995) at B0184-B0185. This technology uses fluorescently-labeled probes targeting specific SNPs as a simple variation of the primers used in standard PCR. Because the amplification of DNA can be detected and quantified in real time, i.e., as the PCR assay progresses, this methodology is often called “real-time PCR” or “quantitative PCR.” Thus, methods of SNP-typing using fluorescent TaqMan probes and real-time PCR were established almost a decade before the filing date of the Patents.

92. I have significant personal experience using these standard and routine arrays for genotyping prior to 2009. For example, I was involved in the early development of DNA microarrays while at Stanford University and was hired by TIGR in 1997 in large part to establish and lead a large-scale effort to establish and use microarrays to understand gene function. My group developed DNA microarray technology for profiling gene expression in humans, mouse, rat, zebrafish, many bacterial species, other mammalian species, plant species including *Arabidopsis*, maize, tomato, potato, rice, and eukaryotic parasites such as *Plasmodium falciparum*, the causative agent of malaria. As part of my recruitment to Dana-Farber in 2005, I negotiated access to commercial microarrays including Affymetrix GeneChips™ and Illumina arrays and I was involved in projects that used both gene

expression and SNP genotyping data. In all of these applications, I used standard and routine commercially available array applications for genotyping.

(c) Sequencing Was a Well-Understood, Routine and Conventional Method For Genotyping By 2009

93. Sequencing was also a well-understood, routine and conventional method for genotyping by 2009. For example, the written description states that:

Genotyping donor and recipient nucleic acids, and/or detection, identification and/or quantitation of the donor-specific nucleic acids after transplantation (e.g. polymorphic markers such as SNPs) can be performed by sequencing such as whole genome sequencing or exome sequencing. Sequencing can be accomplished through classic Sanger sequencing methods which are well known in the art.

B0001-B0024 ('652 Patent) at B0017, 15:1-8. The Patents also explain that:

Genotyping of donor and recipient can establish a single nucleotide polymorphism (SNP) profile for detecting donor DNA. Shotgun sequencing of cell-free DNA in plasma, with analysis of observed unique SNPs, allows quantitation of % donor DNA.

Id. at B0018, 17:32-36; *see also id.* at B0019, 20:31-36 (“Genotyping of the transplant donor and/or the transplant recipient may be performed by any suitable method known in the art including those described herein such as sequencing, nucleic acid array or PCR. In some embodiments, genotyping of the transplant donor and/or the transplant recipient is performed by shotgun sequencing.”).

94. I have significant personal experience using these standard and routine multiplex / high-throughput sequencing techniques for genotyping prior to November 6, 2009. For example, when I began working in the biological sciences at

the Salk Institute in 1992, the first molecular biology techniques that I learned were PCR and Sanger DNA sequencing. As part of my move to Stanford University in 1994, I was tasked with developing a new large-scale DNA sequencing method based on PCR mapping of transposon insertions. My recruitment to the Dana-Farber Cancer Institute in 2005 was largely based on the expectation that high throughput DNA sequencing would soon generate unprecedented quantities of data that could be used to understand cancer. And that expectation very quickly was realized throughout the genomics field.

95. Due to the rapid explosion in use of sequencing-by-synthesis instruments including the 454 sequencer in 2005, the Illumina Genome Analyzer and Applied Biosystems SOLiD sequencer in 2006, and others shortly thereafter, my group and I quickly became involved in multiplex / high-throughput (or Next Generation Sequencing (“NGS”)) DNA sequencing. By 2009, my group and I were routinely analyzing DNA sequence data generated on nearly every multiplex or high-throughput (*e.g.*, NGS) platform available at the time.

96. In addition, in 2008, while a scientific advisor at Helicos Biosciences, I wrote a scientific paper with scientists at Helicos exploring applications of high throughput DNA sequencing entitled “What would you do if you could sequence everything?” that reported on a wide variety of applications, including genotyping.

B0251-B0259 (Kahvejian 2008). By 2009, I was routinely using multiplex / high-throughput sequencing for a wide variety of applications including genotyping.

**(d) The Patents Claim No Improvements To Or
Nonconventional Uses Of The Conventional
Genotyping Methods Available In 2009**

97. The claims recite no improvements to these common approaches to “genotyping,” nor any novel way of carrying them out; rather the Patents explain that the “existing genotyping platforms known in the art” can be employed, handling up to hundreds of thousands of heterozygous and homozygous “[u]sable SNPs,” and note that “[c]ompanies ... currently offer both standard and custom-designed TaqMan probe sets for SNP genotyping that can in principle target any desired SNP position for a PCR-based assay.” B0001-B0024 (’652 Patent) at B0016, 13:58-64. Consistent with the disclosure of the Patents, and my own experience as a researcher in the genomics field in 2009, a POSA understood “genotyping,” including using “selective amplification ... by ... PCR,” and establishing “polymorphism” or “SNP” “profiles” including homozygous or heterozygous variants to be a routine and conventional practice.

98. A declaration submitted to the USPTO during prosecution of the ’652 Patent further confirms that the recited genotyping limitations were routine and conventional as of 2009. In order to overcome a rejection by the patent examiner, a scientist who worked in named inventor Stephen Quake’s Stanford laboratory

submitted a declaration, dated January 30, 2014, to “demonstrate that genotype information obtained from a transplant recipient can be used to establish a polymorphism profile to detect donor-derived cell-free nucleic acids in a sample from the transplant recipient.” B0260-B0263 (Beausang Dec.). In his declaration, the scientist, Mr. John Beausang, describes an analysis he had performed using the claimed methods. *Id.* He states that “[g]enotypes of the transplant recipients were obtained from genomic DNA using the Illumina Omni1-Quad Beadchip following standard protocols.” *Id.* I am familiar with the Illumina BeadChip, which as discussed above in paragraph 89, was available by 2002 and was routinely used by researchers for genotyping to obtain polymorphism, including SNP, profiles following standard protocols by 2009.

3. Performing Multiplex Or High-Throughput Sequencing Of (Or Digital PCR On) The cfDNA To Detect The Genotyped Polymorphisms (Or SNPs)

99. Claim element 1(c) of the '652 Patent recites “multiplex sequencing of the cell-free nucleic acids in the sample followed by analysis of the sequencing results using the polymorphism profile.” Claim element 1(d) of the '497 Patent recites “determining an amount of donor-specific cell-free nucleic acids by ... high-throughput sequencing or [dPCR].” Claim element 1(d) of the '607 Patent recites “performing a high throughput sequencing reaction ... compris[ing] ... sequencing-by-synthesis.” These also were standard techniques for analyzing nucleic acids by

the time of the November 6, 2009 filing date, and they were routinely used by that time to analyze cell-free nucleic acids in samples.

(a) Background On Multiplex / High Throughput Sequencing Technology

100. As explained above, methods for determining the exact sequences of the bases in a stretch of DNA – called sequencing – were well known as of November 6, 2009. *See, e.g.*, B0001-B0024 ('652 Patent) at B0017, 15:6-8. Methods for simultaneously sequencing multiple stretches of DNA in a high-throughput instrument – called multiplex or high-throughput sequencing, also known as Next Generation Sequencing or “NGS” – also were well known and routinely used as of November 6, 2009. *See, e.g., id.* at B0017, 15:8-16:41. The terms “multiplex sequencing” as recited in the '652 Patent, and “high-throughput sequencing” as recited in the '497 and '607 Patents, were used interchangeably by POSAs in the field of nucleic acid analysis in 2009 when the Patents were filed. These terms were understood by POSAs (both in 2009 and now) to include parallel sequencing methods using automated instrumentation.

101. “Sequencing-by-synthesis,” as recited in the '607 Patent claims, is a sequencing technique that by 2009 was one of the multiplex or high-throughput sequencing techniques most routinely used in the field of nucleic acid analysis. It involves determining the sequence of a particular nucleic acid by synthesizing a strand complementary to it inside of a sequencing machine. In the sequencing-by-

synthesis reaction, the bases being added are “tagged” in an identifiable manner, such that the sequencing machine can tell what base is being added at each position, and therefore identify the complete sequence of the nucleic acid that is being sequenced. Because nucleic acids, particularly DNA, form double-stranded structures through complementary binding and specific base-pairing, if a POSA knows the sequence of the strand that is newly sequenced, she can deduce the sequence of the complementary strand being interrogated.

102. The specification also describes a “shotgun” sequencing approach for sequencing circulating nucleic acids. *Id.* at B0014, 9:8-14. (“Detection, identification and/or quantitation of the donor-specific markers (e.g. polymorphic markers such as SNPs) can be performed using real-time PCR, chips (e.g., SNP chips), high-throughput shotgun sequencing of circulating nucleic acids (e.g. cell-free DNA), as well as other methods known in the art including the methods described herein.”). Shotgun sequencing is a general strategy that was developed in the 1980s. B0264-B0274 (Hutchison) at B0268; B0275-B0285 (Green 2001) at B0276, B0281, B0282. In shotgun sequencing, multiple overlapping “reads” for the target DNA are obtained by performing several rounds of sequencing of DNA fragments. After all the fragments are sequenced, computer programs use the overlapping ends of different reads to assemble them into a continuous sequence.

(b) Commercially Available Multiplex And High-Throughput Sequencing Platforms As Of 2009

103. Multiplex and high-throughput sequencing techniques, including sequencing-by-synthesis techniques, had been in use for more than a decade before the 2009 filing date of the Patents. For example, a method called pyrosequencing, which performs sequencing-by-synthesis, was developed and first published in 1993. B0286-B0290 (Nyren 1993); *see also* B0291-B0300 (Ronaghi 2001). It was the foundation for different companies. PYROSEQUENCING AB, out of Uppsala Sweden, launched its first commercial automated pyrosequencing instrument in 1999. B0694-B0903 (Marsh 2007) at B0713. Another is called 454 Life Sciences, Inc. (later acquired by Roche Diagnostics, Inc.). *See also* B0001-B0024 ('652 Patent) at B0017, 15:38-45 (disclosing using 454 sequencers as embodiments of the claimed methods); B0309-B0312 (Coombs) at B0311. 454 Life Sciences commercially launched its first sequencing instruments in 2005. B0301-B0308 (Heather 2016) at B0303; B0309-B0312 (Coombs) at B0311. Based on the machines made available by Pyrosequencing, by the year 2000, pyrosequencing had established itself as a standard and conventional means for multiplex or high-throughput sequencing, including for genotyping SNPs. *See, e.g.*, B0313-B0320 (Ahmadian 2000 at B0313, B0318); B0074-B0079 (Nordstrom 2000) at B0074, B0078; B0291-B0300 (Ronaghi 2001) at B0292-B0293.

104. Another high throughput sequencing-by-synthesis method developed by a group out of Cambridge University in 1998 became the foundation for a company called Solexa (later acquired by Illumina, Inc.). Solexa commercially launched its first sequencing instrument, the “Genome Analyzer,” in 2006. B0321-B0323 (Illumina, History of Sequencing by Synthesis, available at <https://www.illumina.com/science/technology/next-generation-sequencing/illumina-sequencing-history.html>); *see also* B0001-B0024(’652 Patent) at B0017, 15:53-60; B0017-B0018,16:57-17:13 (disclosing using Solexa instruments including the Genome Analyzer as embodiments of the claimed method). As the group that developed this technology reported in Margulies 2005, the instrument branded as the Genome Analyzer could “sequence 25 million bases, at 99% or better accuracy, in one four-hour run,” B11305-1309 (Margulies 2005) at B1305 (Abstract), and had an average read length of 108 bases, *id.* at B1307, Table 1.

105. Illumina acquired Solexa in 2007. Illumina provides a comprehensive line of products for large scale analysis of genetic variation and biological function, including genotyping and sequencing. In 2007, in a 10K SEC filing, Illumina reported that its “[i]nstrument revenue increased by \$77.6 million over prior year, of which \$68.7 million was due to increased sales of our sequencing systems, particularly the Genome Analyzer and cluster stations.” By the end of 2008, Illumina

reported revenue of \$573 million for its products. B0904-B0994 (Illumina 10K) at B0927. Illumina reported that its “Instrument revenue increased by \$64.8 million over prior year, of which \$63.0 million was due to increased sales of our sequencing systems. This increase in revenue can be primarily attributed to shipments of our second generation Genome Analyzer, the Genome Analyzer II (GAII).” *Id.* at B0935. Illumina’s Genome Analyzer, which was in widespread routine use by 2009, is cited in the written description as an example product capable of performing the claimed laboratory techniques. B0001-B0024 (’652 Patent) at B0017, 16:50-59; B0018, 17:1-12; B0022, 26:47-52; *see also* B0260-B0263 (Beausang Decl.) at B0260-B0261.

106. As another example, a high throughput sequencing method co-developed by the named inventor Dr. Quake became the foundation for his company, Helicos, which was founded in 2003, and on which I was a member of the Scientific Advisory Board. Helicos commercially launched its first sequencing instrument, the HeliScope, in 2008. B0581-B0693 (Helicos 10K 2008); *see also* B0001-B0024 (’652 Patent) at B0017, 15:22-37; B0018, 17:22-28 (disclosing using Helicos instruments as embodiments of the claimed method).

107. The written description also describes the Pico Titer Plate device manufactured by 454 Lifesciences, Inc. as a commercial product for performing the recited sequencing element. *Id.* at B0017, 15:38-46. The Pico Titer Plate device,

available as of 2006, combines conventional PCR amplification with pyrosequencing in a high throughput, automated format. The Pico Titer Plate was one of the first next generation multiplex sequencing instruments developed, and was in common usage as of 2009. B0226-B0243 (Voelkerding 2009) at B0227.

108. The acceleration of nucleic acid sequencing technology before 2009 was incredibly rapid, with authors of a pertinent review article describing:

The capabilities of DNA sequencers have grown at a rate even faster than that seen in the computing revolution described by Moore's law: the complexity of microchips (measured by number of transistors per unit cost) doubles approximately every two years, while sequencing capabilities between 2004 and 2010 doubled every five months. The various offshoot technologies are diverse in their chemistries, capabilities and specifications, providing researchers with a diverse toolbox with which to design experiments.

B0301-B0308 (Heather 2016) at B0305; *see also* B0324-B0333 (Morozova (2008) at B0324 ("A new generation of sequencing technologies, from Illumina/Solexa, ABI/SOLiD, 454/Roche, and Helicos, has provided unprecedented opportunities for high-throughput functional genomic research. To date, these technologies have been applied in a variety of contexts, including whole-genome sequencing, targeted resequencing, discovery of transcription factor binding sites, and noncoding RNA expression profiling."); *id.* at B0331 (noting that the commercially available high throughput sequencers "have provided genome-scale sequencing capacity to individual laboratories").

109. In an article published in March 2009, named inventor Dr. Quake reported a study on sequencing using the 454 FLX and Solexa DNA platforms, both of which are described as commercially available in the Patents. B0334-B0345 (White 2009) at B0334; *see also* B0001-B0024 ('652 Patent) at B0017, 15:38-60. In that publication, Dr. Quake states that high-throughput sequencing technologies such as 454 and Solexa that are “based on sequencing by synthesis and sequencing by ligation are revolutionizing biology, biotechnology, and medicine,” and are a “key advance facilitating higher throughput and lower costs for several of these platforms was migration from the clone-based sample preparation used in Sanger sequencing to the massively parallel clonal PCR amplification of sample molecules on beads (Roche 454 and ABI Solid) or on a surface (Solexa).” B0334-B0345 (White 2009) at B0334.

110. By November 2009, POSAs also routinely used multiplex or high-throughput sequencing to detect SNPs in samples. This is confirmed, for example, in a scientific paper by Shen *et al.*, B0346-B0354 (Shen 2010), published online on December 17, 2009, titled “A SNP Discovery Method To Assess Variant Allele Probability From Next-Generation Resequencing Data.” Shen *et al.* explain that “[i]n recent years, next-generation sequencing (NGS) technologies have propelled the rapid progress of genomics studies []. Continuous improvement in NGS technologies are increasing the throughput while lowering costs, thus enabling ultra-

large-scale sequencing efforts.” *Id.* at B0346. The authors explain that [c]urrently, there are several methods available for detecting SNPs from NGS data,” listing several examples, and further explain that they have provided a “freely available software package, Atlas-SNP2” to reduce error rates in interpreting sequencing data. *Id.* at B0346-B0347.

111. Based on my review of the literature and my own experience as a researcher active in cfDNA analysis in 2009, there were a number of commercially available sequencers from which to choose to carry out what the Patent claims describe at a high level as “multiplex” or “high-throughput” “sequencing,” including what the Patents describe as “sequencing-by-synthesis.”

112. The Patents’ disclosure acknowledges these numerous commercial providers of multiplex or high-throughput sequencing instruments as of the time the Patents were filed. For example, the written description states that:

Detection, identification and/or quantitation of the donor-specific markers (e.g. polymorphic markers such as SNPs) can be performed using real-time PCR, chips (e.g., SNP chips), ***high-throughput shotgun sequencing*** of circulating nucleic acids (e.g. cell-free DNA), ***as well as other methods known in the art including the methods described herein.***

B0001-B0024 (’652 Patent) at B0014, 9:8-14 (emphasis added). The Patents disclose, under the heading “Sequencing,” *id.* at B0017, 15:1, numerous examples of commercial products that can carry out the multiplex or high-throughput

sequencing (including sequencing by synthesis), including those discussed above, such as:

- “technology available by Helicos Biosciences Corporation [] such as the Single Molecule Sequencing by Synthesis (SMSS) method,” *id.* at B0017, 15:22-25;
- “technology available by 454 Lifesciences,” *id.* at B0017, 15:39;
- “fiber optics detection [as] described in Marguiles, M., et al.,” *id.* at B0017, 15:46-52;
- “Clonal Single Molecule Array (Solexa, Inc.) or sequencing-by-synthesis (SBS) utilizing reversible terminator chemistry,” *id.* at B0017, 15:53-56;
- “AnyDot-.chips (Gonovox, Germany),” *id.* at B0017, 15:61-65; and
- “sequencers such as the Illumina Genome Analyzer,” *id.* at B0017, 16:57-59.

113. Furthermore, a declaration submitted by Mr. Beausang to the USPTO during prosecution of the '652 Patent, in order to overcome rejections by the patent examiner, further confirms that the multiplex and high-throughput sequencing limitations were routine and conventional as of 2009. In his declaration, Mr. Beausang states that in carrying out the method recited in the '652 Patent claims, “[s]equencing libraries were constructed from cell-free DNA using commercially available kits and sequenced following standard protocols using an Illumina GAII sequencer.” B0260-B0263 (Beausang Decl.) at B0260-B0261. I am familiar with the Illumina GAII sequencer, which was routinely used in 2009 by researchers, including myself, using commercially available kits and standard protocols.

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**(c) Sensitivities And Error Rates Inherent In The
Available Sequencing Instruments**

114. The Patent claims further recite certain levels of sensitivity in the claimed methods, for example at '652 Patent claim element 1(d), which recites “wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)”¹³; and at '497 Patent claim element 1(d) and '607 Patent claim element 1(f), which recite detection when the donor-specific circulating cell-free nucleic acids make up “at least 0.03% of the total circulating cell-free [nucleic acids/[DNA]]” in the sample. These sensitivity levels are not improvements over the methods already available, nor do the Patents disclose or claim any nonconventional use of the available methods in order to achieve a particular sensitivity. Rather, the recited sensitivities

¹³ I do not believe a POSA would understand the meaning of the phrase “wherein sensitivity of the method is greater than 56% compared to sensitivity of current surveillance methods for cardiac allograft vasculopathy (CAV)” of '652 Patent claim element 1(d), as it makes no sense in the context of the claims. For example, “sensitivity” can have different meanings and there are different ways to measure it depending on what it refers to. For example, there is a level of “sensitivity” inherent in the sequencing methods recited in the claims that can be measured in one of several different ways based on the outcome of the sequencing instrument. There is also a clinical “sensitivity” inherent in the method based on how accurate the method is in identifying those patients who have organ transplant rejection or failure. The claim does not convey to a POSA what “sensitivity” it refers to or how it is measured. Notwithstanding, regardless of what the “sensitivity” in the claim refers to or how it is measured, it is an inherent feature of the claimed methods and neither the claims nor the written description refer to any improved or nonconventional way of achieving a specific level of “sensitivity” beyond what inherently results from the use of the conventional methods recited.

are inherent features of standard use of the methods, which themselves are routine and conventional.

115. For example, as to '652 Patent claim element 1(d), the Patents' written description explains that "[t]he invention provides methods that are sensitive and specific," and "[i]n some embodiments, the methods described herein have at least 56% sensitivity." B0001-B0024 ('652 Patent) at B0021, 23:31-36. A POSA would understand this is an inherent feature of the recited method, based on the conventional techniques recited. The Patents' written description states that "[t]he practice of the present invention employs, unless otherwise indicated, conventional techniques ... which are within the skill of the art. ..." *Id.* at B0012, 5:36-49. The written description does not anywhere indicate that the methods are practiced in a nonconventional way to achieve greater than 56% sensitivity compared to sensitivity of then-current surveillance methods for CAV – or compared to any other metric for that matter.

116. Furthermore, to the extent the element "sensitivity of the method is greater than 56%" ('652 Patent claim element 1(d)) refers to the sensitivity of a clinical diagnosis, this would be an inherent outcome of practicing the routine, conventional method steps. In a clinical diagnosis, "sensitivity" refers to the ability of the test to correctly identify those with the condition – here transplant rejection or failure (*i.e.*, true positive rate). There is nothing inventive about reciting a sensitivity

greater than 56% compared to anything. As far as the claims are concerned, a sensitivity of greater than 56% is slightly better odds than flipping a coin in terms of diagnosing transplant rejection or failure. Indeed, even a test that identifies every transplant recipient as undergoing transplant rejection or failure would have a 100% sensitivity rate (because the group would include all patients undergoing rejection/failure)--but the test would be useless because it would not eliminate false positives (it would also include patients who were not actually undergoing rejection/failure). The written description contains no explanation of a nonconventional way of achieving even 56% sensitivity (which, in my opinion, would be ineffective at diagnosing transplant rejection or failure as the claims purport in any event).

117. The written description only describes at a high level “sensitive and non-invasive methods, devices, compositions and kits for monitoring organ transplant patients, and/or for diagnosing or predicting transplant status or outcome (e.g. transplant rejection),” and the lowest sensitivity described in the patent is 56%. B0001-B0024 ('652 Patent) at B0021, 23:32-36 (“In some embodiments, the methods described herein for diagnosing or predicting transplant status or outcome have at least 56%, 60%, 70%, 80%, 90%, 95% or 100% sensitivity. In some embodiments, the methods described herein have at least 56% sensitivity.”). The written description does not indicate any nonconventional technique for arriving at

this (albeit clinically ineffective) sensitivity for diagnosis – to the contrary it lacks any explanation as to how this sensitivity is achieved. Accordingly, a POSA would know that a sensitivity of at least 56% would have to be (and is) an inherent result of using the claimed techniques.

118. As to sensitivity of the sequencing methods, including as recited in '497 Patent claim element 1(d) and '607 Patent claim element 1(f), wherein detection occurs when the donor-specific circulating cell-free nucleic acids make up “at least 0.03% of the total circulating cell-free [nucleic acids/[DNA]]” in the sample, the Patents’ written description states that “[h]igher sensitivity can be achieved simply by sequencing more molecules, i.e., using more channels.” B0001-B0024 ('652 Patent) at B0018, 17:12-13. The written description also explains that, for example, “[c]urrently, sequencers such as the Illumina Genome Analyzer have read lengths exceeding 36 base pairs,” *id.* at B0017, 16:57-59, and that “[o]n the Genome Analyzer ... If one wants to establish a lower limit of sensitivity for this method by requiring at least 100 donor molecules to be detected, then it should have a sensitivity capable of detecting donor molecules when the donor fraction is as low as 0.03%.” *Id.* at B0018, 17:1-11. This is merely states, again, an inherent feature of the conventional sequencing instruments, in standard use, as recited in the claims.

119. Indeed, it was well-known in the art by November 6, 2009 that “[h]igher sensitivity can be achieved simply by sequencing more molecules,” as the

written description states. *See* B0001-B0024 ('652 Patent) at B0018, 17:12-13. The written description acknowledges that “[t]here are two components to sensitivity: (i) the number of molecules analyzed (depth of sequencing) and (ii) the error rate of the sequencing process.” *Id.* at B0017, 16:52-55. POSAs prior to November 6, 2009 were well aware that sequencing more molecules, or increasing sequencing depth, improves sensitivity. As Voelkerding *et al.* explained in a 2009 review article reviewing multiplex / high-throughput (NGS) sequencing technology: “Accuracy in NGS is achieved by sequencing a given region multiple times, enabled by the massively parallel process, with each sequence contributing to ‘coverage’ depth.” B0226-B0243 (Voelkerding 2009) at B0236. In another example, Thomas *et al.* in 2006 noted that “[s]equence variants that represent a fraction of a complex sample can be vastly oversampled, thus enabling statistically meaningful quantification of low-abundance variants.” B1301-1304.1 (Thomas 2006) at B1301. This is further demonstrated in Thomas 2006 at Supplemental Figure 2, which shows that the detection limit is determined by sequencing error rate and sequencing read depth (*i.e.*, how many molecules are sequenced), reflecting the fact that there was a well-known correlation between the number of molecules sequenced and sensitivity including limit of detection of donor fraction. *Id.* at B1304.1.

120. The '607 Patent, at claim element 1(d), also recites that “said sequencing-by-synthesis reaction has a sequencing error rate of less than 1.5%.”

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This, too, is a feature inherent in common methods of using the sequencing instruments disclosed in the written description. In the context of the Patents, the sequencing error rate is an estimate of the proportion of all bases (often expressed as a decimal) sequenced by a sequencer that are incorrect. These errors can be caused by a combination of problems, for example, during sample preparation, misidentification of single base additions by the sequencer, background noise, or some combination of these and other factors. The Patents' written description explains that "it is possible to systematically lower the sequencing error rate by resequencing the sample template multiple times, as has been demonstrated by Helicos BioSciences (Harris, T.D. et al., Science, 320, 106-109 (2008))." B0001-B0024 ('652 Patent) at B0018, 17:22-26. The written description further explains that, as to the numerous commercial sequencing platforms disclosed in the patents, "[t]ypical sequencing error rates for base substitutions vary between platforms, but are between 0.5-1.5%." *Id.* at B0018, 17:20-21. Thus, as the Patents' written description recognizes, error rates were inherent in the recited sequencing techniques, and methods for lowering sequencing error rates were already known and established in the field before the Patents were filed.

121. The literature as of 2009 confirms that the recited sensitivity levels were inherent in the conventional use of the sequencing instruments at the time. For example, in a paper co-authored by Patent inventor Stephen Quake, Harris *et al.*,

Science (2008) 320:106-109, B0355-B0359, the authors explain the science behind the inherent sequencing error rates in Helicos sequencing technology. The authors also describe inherent error rates significantly lower than 1.5%. *Id.* at B0357.

(d) Digital PCR Methods Were Routine And Commercially Available In 2009

122. '497 Patent claim element 1(d) recites, as an alternative to high-throughput sequencing, a technique called digital PCR, or "dPCR." This technique also was known and conventional as of 2009. For example, the written description cites to and incorporates by reference a 2006 publication disclosing its use, at B0001-B0024 ('652 Patent) at B0016, 14:58-67.

(e) The Patents Claim No Improvements To Or Nonconventional Uses Of The Conventional Multiplex/High-Throughput Sequencing Or dPCR Methods Available In 2009

123. The "multiplex sequencing" and "high-throughput sequencing" (and "dPCR") techniques recited in the claims of the Patents refer to a broad range of well-established and routine parallel sequencing technologies, including sequencing-by-synthesis, as of 2009. The Patents recite using them only at a high level of generality, and do not purport to improve on them or claim any nonconventional use of them.

4. Quantifying The Transplant cfDNA In The Sample, Using The Polymorphisms / SNPs In The Sequences

124. Claim element 1(d) of the '652 Patent recites “determining a quantity of the donor cell-free nucleic acids based on the detection of [donor and recipient cell-free nucleic acids] by the multiplexed sequencing.” Claim element 1(d) of the '497 Patent recites “determining an amount of donor-specific cell-free nucleic acids ... by detecting a homozygous or a heterozygous SNP within the [donor cell-free nucleic acids].” Claim element 1(f) of the '607 Patent recites “quantifying an amount of [transplant cfDNA] ... using markers distinguishable between ... recipient and ... donor ... wherein said markers ... comprise [SNPs] selected from said at least 1,000 [SNPs].” These quantification steps are broadly recited with no further specificity. A POSA would have understood that they could employ any of a wide range of standard techniques for quantifying nucleic acids, which were already well known and established by the filing date of the Patents.

125. The Patents' written description explains that “[d]etection, identification, and/or quantification of the donor-specific markers (e.g., polymorphic markers such as SNPs) can be performed using real-time PCR, chips (e.g., SNP chips), high-throughput shotgun sequencing of circulating nucleic acids (e.g. cell-free DNA), as well as other methods known in the art including the methods described herein.” B0001-B0024 ('652 Patent) at B0014, 9:8-14. This is also described elsewhere in the written description, for example *id.* at B0020, 21:5-9,

which states that “[t]he presence or absence of one or more nucleic acids from the transplant donor in the transplant recipient may be determined by any suitable method known in the art including those described herein such as sequencing, nucleic acid arrays or PCR.”

126. Quantification of SNPs based on multiplex or high-throughput sequencing data was a well-known and routine practice by the time of the 2009 filing date of the Patents. I, myself, was researching cancer-related polymorphisms, including SNPs, present in cfDNA in cancer patients using high-throughput sequencing data at that time. For example, my position at Dana-Farber involved contributing to developing analytic methods for the processing, including quantification, of DNA sequencing data generated by multiplex or high-throughput sequencing. This included analysis, including quantification, of sequencing data from cancer cell lines, bulk tumor samples, circulating tumor cells, and cell-free nucleic acids. Using multiplex or high-throughput sequencing to generate quantitative sequencing data, including from cfDNA, was a routine practice by November 6, 2009, and I used standard techniques for generating such sequencing data in my own experience.

127. By November 2009, POSAs were routinely publishing data generated by multiplex or high-throughput sequencing to quantify SNPs, including for the purpose of detecting different genotypes in a sample. For example, in a scientific

paper published by Sampson and Zhao, B0360-B0388 (Statistical Applications in Genetics and Molecular Biology (2009) 8(1):1-27, titled “Identifying Individuals in a Complex Mixture of DNA with Unknown Ancestry”), the authors described use of high-throughput sequencing data, including as generated from “Illumina” instruments, to “determine whether a specific individual contributes DNA” to “a mixture of DNA samples from numerous individuals.” B0360-B0388 (Sampson 2009) at B0360-61. The authors provide algorithms for determining “allele frequencies,” which a POSA would understand describes determining quantities of SNPs, from this data. *Id.* at, *e.g.*, B0362. The authors further examine and compare SNP frequency data generated on high-throughput sequencing platforms, such as Illumina’s, to such data generated on other types of platforms. *Id.* at B0368; *see also id.* at B0361 (explaining the platforms being tested are “e.g. Illumina, Affymetrix”).

128. The claims of the Patents recite quantifying donor cell-free nucleic acids only at a high level, and do not specify any particular technique for doing so. The claims do not purport to provide any innovative or nonconventional method for quantifying polymorphisms or SNPs in cell-free nucleic acids based on sequencing data. Nor do the claims purport to claim any improvement over the existing means for doing so as of 2009.

C. The Claimed Combinations Of Steps For Detecting Multiple Genomes In A cfDNA Sample Were Conventional

1. The Patents' Written Description States That The Laboratory Techniques In The Claims As A Whole Were Conventional As Of 2009

129. As I note above, the Patents' written description states that “[t]he practice of the present invention employs, *unless otherwise indicated, conventional techniques* of immunology, biochemistry, chemistry, molecular biology, microbiology, cell biology, genomics and recombinant DNA, *which are within the skill of the art.*” B0001-B0024 (’652 Patent) at B0012, 5:36-40 (emphasis added). I did not find any indication in the Patents that the claimed combination of laboratory techniques was nonconventional, or carried out in a way that modifies or improves upon the traditional combination of these laboratory techniques.

130. To the contrary, the Patents' written description states that the combination of elements in the claims was routine and carried out using commercial products without modification at the time of the Patents' filing date. For example, the Patents' written description states that “[g]enotyping¹⁴ donor and recipient nucleic acids, and/or detection, identification and/or *quantification of the donor-specific nucleic acids*¹⁵ after transplantation (e.g. *polymorphic markers such as*

¹⁴ ’652 Patent claim element 1(b); ’497 Patent claim elements 1(a) and (b); ’607 Patent claim element 1(c).

¹⁵ ’652 Patent claim element 1(d); ’497 Patent claim element 1(d) ; ’607 Patent claim element 1(f).

SNPs)¹⁶ *can be performed by sequencing*¹⁷ such as whole genome sequencing or exome sequencing.” B0001-B0024 (’652 Patent) at B0017, 15:2-6 (emphasis added). The written description, in the same section, identifies several commercial multiplex and high-throughput sequencing¹⁸ platforms routinely used to carry out this combination of steps by the 2009 filing date. *Id.* at B0017-B0018, 15:22-17:28 (further disclosing the inherent sensitivities and error rates inherent in the standard use of the sequencing equipment); see also section VI.B above.

131. The written description identifies obtaining or providing a sample of cell-free nucleic acids¹⁹ as routine and conventional, and does not identify any nonconventional way of obtaining a sample that is used with the claimed combination. *See* section VII.B. above.

132. As to the ’607 Patent, the written description does not indicate anything nonconventional about “selective amplification of ... at least 1,000 [SNPs] ... by PCR,” as recited in claim element 1(c), or its combination with high throughput sequencing as recited in elements 1(d) and (e) to quantify the cfDNA as recited in

¹⁶ ’652 Patent claim element 1(b); ’497 Patent claim elements 1(a) and (b) ; ’607 Patent claim element 1(f).

¹⁷ ’652 Patent claim element 1(c); ’497 Patent claim elements 1(d) ; ’607 Patent claim elements 1(d) and (e).

¹⁸ ’652 Patent claim element 1(c); ’497 Patent claim elements 1(d) ; ’607 Patent claim element 1(d).

¹⁹ ’652 Patent claim element 1(a); ’497 Patent claim elements 1(c) ; ’607 Patent claim elements 1(a) and (b).

element 1(f). Rather, again, the written description explains that “[g]enotyping donor and recipient nucleic acids, and/or detection, identification and/or *quantitation* of the donor-specific nucleic acids after transplantation (e.g. *polymorphic markers such as SNPs*) *can be performed by PCR*.” B0001-B0024 (’652 Patent) at B0016, 14:29-32 (emphasis added). The written description describes commercially available PCR-based products that were routinely used to genotype and quantitate SNPs as of 2009, stating that with these products, “[u]sable SNPs may comprise approximately 500,000 heterozygous donor SNPs and approximately 160,000 homozygous donor SNPs,” and that “[c]ompanies ... currently offer both standard and custom-designed TaqMan probe sets for SNP genotyping that can in principle target any desired SNP position for a PCR-based assay.” *Id.* at B0017, 13:55-64.

133. Moreover, the written description does not indicate anything nonconventional about selectively amplifying 1,000 SNPs (or even 10,000 SNPs as recited in dependent claim 5) and combining that with high-throughput sequencing of the selectively amplified DNA. Indeed, contemporaneous literature indicates that was conventional, and numerous commercial products were available to perform it. *See, e.g.* B0224-B0225 (RainDance 2008) (describing commercial product for amplifying thousands of SNPs and combining with subsequent high-throughput sequencing by synthesis); B0226-B0243 (Volkerding 2009) (review article

describing several different products for “targeted genomic resequencing,” including to detect “polymorphisms,” that involve up to hundreds of thousands of capture probes to “enrich[]” for the target polymorphisms, “with the enriched DNA amplified by PCR before NGS [*e.g.*, high-throughput sequencing] library preparation”).

134. In fact, the written description does not mention sequencing of selectively amplified cfDNA at all – there is no reference to it in the specification. The written description does not indicate that the claimed method performs this combination by modifying the recited laboratory techniques or using them in a nonconventional way, nor does it state this is an improvement over the methods the written description otherwise describes as conventional. *See* B0001-B0024 (’652 Patent) at B0012, 5:36-40 (“The practice of the present invention employs, unless otherwise indicated, conventional techniques ... which are within the skill of the art.”).

135. Thus, each of the combinations of the claims of the Patents as a whole is described in the written description as conventional and routinely performed, including by commercial instruments, as of the Patents’ filing date.

2. POSAs Have Long Recognized That The Same Methods For Detecting Fetal, Cancer, And Infectious cfDNA Could Be Used To Detect Transplant cfDNA

136. By 2009, the combinations of laboratory techniques claimed in the Patents were well understood, routine and conventional methods for quantifying cfDNA having different genotypes in a biological sample. Other scientists including myself had established, and were routinely using, the claimed combination of laboratory techniques for quantifying fetal cfDNA in samples from a pregnant woman, cancer cfDNA in samples from a cancer patient, and bacterial or viral cfDNA in samples from an infected patient or an infected animal, among other applications, as I discuss in more detail below. POSAs prior to 2009 stated that these same methods could be used to detect and quantify cfDNA from any of these sources, and acknowledged that they also applied to detecting transplant donor-derived cfDNA in an organ transplant recipient's sample.

137. Indeed, the Patents' written description recognizes that common approaches were being applied broadly to study transplant rejection/failure, pregnancy, cancer, and other conditions in which cell-free nucleic acids representing different genotypes are present in a patient's circulation. *See, e.g.*, B0001-B0024 ('652 Patent) at B0012, 6:67-7:5; *id.* at B0013, 7:16-19 (explaining through examples that "results collectively establish both circulating DNA, either free in plasma or from circulating cells, as a useful species in cancer detection and

treatment”); *id.* at B0013, 7:19-23 (explaining with examples that “[c]irculating DNA has also been useful in healthy patients for fetal diagnostics, with fetal DNA circulating in maternal blood serving as a marker for gender, rhesus D status, fetal aneuploidy, and sex-linked disorders”); *id.* at B0013, 8:1-21 (explaining with examples that “results establish that for heart transplant patients, donor-derived DNA present in plasma can serve as a potential biomarker for the onset of organ failure”). As the written description explains, and a POSA in the relevant November 2009 period appreciated, the approaches to detecting and quantifying foreign or abnormal cell-free nucleic acids could be broadly applied to many natural phenomena:

In all these applications of circulating nucleic acids, the presence of sequences differing from a patient's normal genotype has been used to detect disease. In cancer, mutations of genes are a tell-tale sign of the advance of the disease; in fetal diagnostics, the detection of sequences specific to the fetus compared to maternal DNA allows for analysis of the health of the fetus. ... [A]s cell-free DNA or RNA often arises from apoptotic cells, the relative amount of donor-specific sequences in circulating nucleic acids should provide a predictive measure of on-coming organ failure in transplant patients...

B0001-B0024 (’652 Patent) at B0013, 7:30-46.

138. Consistent with the Patents’ disclosure, the application of methods for detecting fetal and cancer cell-free nucleic acids to detecting analogous transplant-related natural phenomena was well-accepted among POSAs. The named inventors of the Patents have acknowledged this not only in the Patents, but also in other

publications. For example, in 2011 Stanford published a report in one of its newsletters quoting two of the named inventors – Steven Quake and Hannah Valantine. They explained that the application of known methods for detecting fetal cfDNA to the analogous phenomenon of transplant cfDNA was the basis for their idea underlying the Patents. As the Stanford newsletter reported:

The current study began when Valantine noticed research by Quake in 2008 showing that it is possible to detect fetal chromosomal abnormalities by sequencing cell-free DNA fragments in a maternal blood sample.

“When I saw that, I thought, wow, this technique could probably be used to monitor heart rejection,” said Valantine, *noting that cells damaged during rejection also release DNA into the circulatory system.*

“Hannah sought me out and I realized that an organ transplant can also be thought of as a genome transplant,” said Quake. *“Someone else’s genome is in your body. So by looking at variations in the DNA sequence, we can identify which DNA segments come from the new heart, and which come from you.”*

B0389-B0391 (<https://med.stanford.edu/news/all-news/2011/03/to-better-detect-heart-transplant-rejections-scientists-test-for-traces-of-donors-genome.html>)
(emphasis added).

139. Similarly, a 2014 article in a Stanford Engineering publication described the Patent inventors’ adaptation of a method for fetal testing into the foundation for the Patents’ methods as follows:

The cell-free DNA technique hinges on the existence in the genome of naturally occurring regions of variation called single nucleotide polymorphisms, or SNPs. In 2008, Hannah Valantine, then a Stanford professor of cardiology, ***realized that a DNA-sequencing technique developed in Quake's lab to pick out small quantities of fetal DNA from a pregnant woman's blood might also be useful to track the fate of a transplanted organ.***

B0392-B0394 (Stanford Engineering 2014) at B0393 (emphasis added).

140. POSAs up to more than a decade before the 2009 filing date of the Patents recognized the ease of applying routine methods for detecting foreign genomes to the detection of transplant cfDNA. For example, in 1998 Lo *et al.* reported in a publication that: “We have shown that DNA from fetuses is present in the plasma of their mothers, and now suggest that, ***in transplant patients, DNA from the organ donor may also be present in the plasma of the recipient.***” B0080-B0081 (Lo 1998) at B0080 (emphasis added). The Lo group further recognized that “[s]ince graft rejection is an important cause of cell death in the transplanted organ, our observations raise the possibility that the concentration of donor DNA in the recipient's plasma may be a marker for rejection.” B0080-B0081 (Lo 1998) at B0080.

141. Thereafter, and following the Human Genome Project's complete sequencing of the human genome in April 2003, methods were quickly developed, standardized, commercialized, and applied by POSAs to detect different genotypes in a single patient sample. *See, e.g.*, B0094-B0155 (2001 Human Genome Project)

at B0098, B0101; B0156-B0183# (Human Genome Project NIH FAQ) at B0158, B0162; B0164-B0183 (1000 Genome Project) at B0164, B168. For example, in 2004, Ding *et al.* published a scientific paper describing laboratory techniques for detecting circulating cell-free nucleic acids that could be broadly applied to conditions involving different genotypes in a sample:

These advances will help in catalyzing the clinical applications of fetal nucleic acids in maternal plasma. This analytical approach also will have *implications for many other applications of circulating nucleic acids in areas such as oncology and transplantation.*

B0395-B0400 (Ding 2004) at B0395t (emphasis added). Ding 2004 also explained that, beyond detecting fetal cfDNA, the same laboratory techniques “could be extended to other areas of circulating nucleic acid analysis, including circulating tumor-specific DNA, such as Epstein–Barr virus DNA in nasopharyngeal carcinoma patients, KRAS point mutations, and donor-specific DNA in transplant recipients.” *Id.* at B0399.

142. Likewise, in a PCT patent application filed on July 9, 2004 and published March 17, 2005, Cantor *et al.* described a method for detecting different genotypes arising from a range of natural phenomena in a sample:

The method allows accurate detection of nucleic acids that are present in very small amounts in a biological sample. For example, the method of the present invention is preferably used to detect fetal nucleic acid in maternal blood sample; circulating tumor-specific nucleic acids in a blood, urine or stool sample; and *donor-specific acids in transplant recipients*. In another embodiment, one can detect viral, bacterial, fungal, or other foreign nucleic acids in biological sample.

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B0995-B1049 (WO 2005/023091) (“Cantor 2005”), at B0995, Abstract (emphasis added).

143. Similarly, in a PCT patent application filed March 26, 2007 and published March 26, 2008, B1050-B1104 (WO2008/118988 A1) (“Ehrich 2008”), Ehrich *et al.* described a laboratory technique for detecting and analyzing a range of different types of circulating cell-free nucleic acids, noting that “[i]n addition to prenatal applications, the methods find utility in a range of applications, including, but not limited to, detecting rare cancer mutations, ***detecting transplant rejection*** and forensics.” B1050-B1104 (WO2008/118988 A1) (“Ehrich 2008”) at B1067, 17:20-22(emphasis added).

144. In another example, Lo *et al.* filed a PCT patent application on July 23, 2008, which published on January 29, 2009, B1105-B1219 (PCT Application No. WO2009/013492) (“Lo PCT 2009”). At paragraph [0003], the Lo group described a method for “determining a nucleic acid sequence imbalance,” which “generally relates to the diagnostic testing of genotypes and diseases by determining ***an imbalance between two different nucleic acid sequences...***” The Lo group noted that the method relates “particularly to the identification of ... mutations and genotypes in a fetus via testing of a sample of maternal blood,” and “also relates to the detection of cancer, ***the monitoring of transplantation***, and the monitoring of infectious diseases.” *Id.* (emphasis added).

145. In all of the above examples from the literature, scientists describe methods for analyzing different nucleic acid sequences, or genotypes, in the same sample. As these examples demonstrate, by the November 6, 2009 filing date of the Patents, POSAs recognized that the same laboratory techniques for detecting different genotypes in a cfDNA sample would apply to a range of natural phenomena, including fetal conditions, cancer, organ transplant monitoring, and infectious disease.

3. POSAs Had Already Established The Claimed Laboratory Techniques By 2009 To Detect Multiple Genotypes In A Sample, Including In The Transplant Setting

146. Confirming the Patents' disclosure of established methods known in the art, numerous prior and contemporaneous publications describe combining the claimed laboratory techniques to detect different genotypes in different contexts, including transplant, fetal, cancer, infectious disease, and others.

147. I provide other non-exhaustive examples here.

(a) Cantor et al. – 2007

148. Cantor *et al.* filed a U.S. Patent Application on February 28, 2006, which published as B0401-B0427 (U.S. 2007/0207466) (“Cantor 2007”) on September 6, 2007. Cantor 2007 describes a method for “*accurate detection of nucleic acids that are present in very small amounts in a biological sample ... preferably used to detect* fetal nucleic acid in maternal blood sample; circulating

tumor-specific nucleic acids in a blood, urine or stool sample; and *donor-specific acids in transplant recipients.*” B0401-B0427 (Cantor 2007) at B0401, Abstract (emphasis added). Cantor 2007 explains the same method also “can detect viral, bacterial, fungal, or other foreign nucleic acids in biological sample.” *Id.*

149. Exemplary disclosures of Cantor 2007 describing the combination of Patent claim elements are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	Cantor described a method of detecting “foreign nucleic acids” “in a biological sample” such as a “blood sample,” which a POSA would understand requires obtaining or providing a biological sample as recited in ’652 Patent claim element 1(a) and ’497 Patent claim element 1(c). <i>See</i> B0401-B0427 (Cantor 2007) at B0401, Abstract. Cantor also disclosed that certain embodiments are “a method for the detection of ... mutations in maternal plasma...,” which requires providing a plasma sample as recited in ’607 Patent claim element 1(a). <i>Id.</i> at B0409, ¶ [0016].
Genotyping to obtain a SNP profile	In an exemplary embodiment in the fetal context, Cantor disclosed that the method comprised “selecting one or more [SNPs],” then “determining the fetal genotype from a sample DNA isolated from the plasma, serum, or whole blood of the pregnant mother ... [by] performing SNP[-specific] ... primer-extension assay in several replicates...” B0401-B0427 (Cantor 2007) at B0411 ¶ [0023]. A POSA would understand this disclosure of Cantor to refer to genotyping to generate a polymorphic, particularly SNP, profile as disclosed in ’652 Patent claim element 1(b) and ’497 Patent claim elements 1(a) and (b). A POSA would further understand Cantor’s disclosure of a “primer-extension assay” to refer to a form of amplification, such as PCR, and disclosure of a SNP-specific primer-extension assay as a form of selective amplification of SNPs by PCR, as recited

	in '607 Patent claim element 1(c). <i>See, e.g., id.</i> at B0409, ¶ [0016] (describing “using methods such as the primer-extension of polymerase chain reaction (PCR) products”).
Multiplex / high-throughput sequencing-by-synthesis	Cantor disclosed numerous commercially available means for detecting the SNPs, including “pyrosequencing techniques (Pyrosequencing, Inc., Westborough, Mass.).” <i>Id.</i> at B0420-B0424 ¶ [0054]; [0063]. A POSA would understand this to be a type of multiplex, or high-throughput sequencing instrument that uses sequencing-by-synthesis, as recited in '652 Patent claim element 1(c), '497 Patent claim element 1(d), and '607 Patent claim elements 1(d) and (e). <i>See id.</i> at B0424, ¶ [0063] (“... PYROSEQUENCING™ (Uppsala, Sweden) [] essentially is sequencing by synthesis.”).
Quantitating the foreign cfDNA	Cantor disclosed “quantitating the fetal nucleic acids or alleles using the methods of the present invention” disclosed in its written description. <i>E.g.,</i> B0401-B0427 (Cantor 2007) at B0415; B0420, ¶ [0036]; [0053]. A POSA would understand the disclosure of quantitating fetal alleles in Cantor to refer to quantifying or determining an amount of foreign cfDNA in the sample as recited in '652 Patent claim element 1(d), '497 Patent claim element 1(d), and '607 Patent claim element 1(f).

150. As this exemplary disclosure of Cantor 2007 demonstrates, a POSA understood that as of the 2006 filing date of Cantor 2007, laboratory techniques were commercially available for carrying out the method of obtaining a sample—genotyping—sequencing—and quantifying cell-free nucleic acids as claimed in the Patents to detect different genotypes, including in the organ transplant context. The Patents do not claim any unconventional use of this combination of laboratory

techniques described in Cantor 2007, nor do they claim any unique way of combining or using the techniques.

(b) Lo et al. – 2009

151. In another example, the Lo group described a method that “relates to the detection of cancer, *the monitoring of transplantation*, and the monitoring of infectious diseases” in a U.S. Patent application filed on July 23, 2008 and published as U.S. 2009/0087847 (“Lo U.S. 2009”). B1220-B1290 (Lo U.S. 2009) at BB1252, ¶ [0003] (emphasis added).

152. Exemplary disclosures of Lo U.S. 2009 describing the combination of Patent claim elements are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	Lo exemplified its disclosed method in an embodiment in which “plasma DNA will be extracted.” B1220-B1290 (Lo U.S. 2009) at B1274, ¶ [0289]. That plasma DNA contains “maternal and fetal DNA in the plasma sample.” <i>Id.</i> A POSA would understand that this disclosure in Lo requires providing or obtaining a biological sample, specifically a plasma sample from the blood, that contains different genotypes as recited in ’652 Patent claim element 1(a), ’497 Patent claim element 1(c), and ’607 Patent claim elements 1(a) and (b), respectively.
Genotyping to obtain a SNP profile	Lo disclosed genotyping generally, at ¶ [0003], stating the disclosed method “generally relates to the diagnostic testing of genotypes and diseases by determining an imbalance between two different nucleic acid sequences...in a fetus...” and “also relates to the detection of cancer, the monitoring of transplantation, and the monitoring of infectious diseases.” Lo described embodiments that evaluate a “clinically relevant

	<p>nucleic acid sequence,” <i>id.</i> at B1254, ¶ [0059], against a “background nucleic acid sequence.” <i>Id.</i> at B1254, ¶ [0060]. Lo described clinically relevant nucleic acid sequences as (among other things) “sequences which are mutated, deleted, or amplified in a malignant tumor, e.g. sequences in which loss of heterozygosity or gene duplication occur.” <i>Id.</i> at B1254, ¶ [0059]. It described background nucleic acid sequences as (among other things) an allele from the same chromosome as a clinically relevant sequence, but nonetheless “distinct due to heterozygosity.” <i>Id.</i> at B1254, ¶ [0060].</p> <p>Lo also disclosed that in an example of the method, the fractional percentage of fetal material is “determined by measuring the amount of a fetal-specific marker (e.g. ... genetic polymorphism markers (e.g. SNPs) ... in relation to a non-fetal-specific marker.” <i>Id.</i> at B1258, ¶ [0100]. Lo disclosed “determining the fractional concentration of fetal DNA ... through the quantification of polymorphic differences between the pregnant woman and the fetus,” citing references disclosing established techniques, and providing “[a]n example of this method ... to target polymorphic sites at which the pregnant woman is homozygous and the fetus is heterozygous.” B1220-B1290 (Lo U.S. 2009) at B1272-B1273, ¶ [0278].</p> <p>A person of skill in the art would understand these disclosures as describing the kind of genotyping used to establish polymorphism or SNP profiles for different genotypes in the sample as recited in ’652 Patent claim element 1(b), and ’497 Patent claim elements 1(a) and (b).</p>
Multiplex / high-throughput sequencing and digital PCR	<p>Lo also described using commercially available digital PCR and multiplex/high-throughput (a.k.a. “massively parallel”) sequencing instruments that were routinely used to carry out the techniques recited in ’652 Patent claim element 1(c), ’497 Patent claim element 1(d), and ’607 Patent claim element 1(d). These Lo disclosures include using “microfluidics digital PCR chips,” citing references dating back to 2006, and “massively parallel genomic sequencing,” citing references</p>

	<p>dating back to 2005. B1220-B1290 (Lo U.S. 2009) at B1263, ¶ [0176]. Lo U.S. 2009 disclosed several specific examples of the routine use of these commercial products, for example:</p> <p>Massively parallel sequencing, such as that achievable on the 454 platform (Roche) (Margulies, M. et al. 2005 Nature 437, 376-380), Illumina Genome Analyzer (or Solexa platform) or SOLiD System (Applied Biosystems) or the Helicos True Single Molecule DNA sequencing technology (Harris TD et al. 2008 Science, 320, 106-109), the single molecule, real-time (SMRT™) technology of Pacific Biosciences, and nanopore sequencing (Soni GV and Meller A. 2007 Clin Chem 53: 1996-2001), allow the sequencing of many nucleic acid molecules isolated from a specimen at high orders of multiplexing in a parallel fashion (Dear BriefFunct Genomic Proteomic 2003; 1: 397-416). Each of these platforms sequences clonally expanded or even non-amplified single molecules of nucleic acid fragments. As a high number of sequencing reads, in the order of hundred thousands to millions or even possibly hundreds of millions or billions, are generated from each sample in each run, the resultant sequenced reads form a representative profile of the mix of nucleic acid species in the original specimen.</p> <p>B1220-B1290 (Lo U.S. 2009) at B1271, ¶ [0262].</p>
Quantitating the foreign cfDNA	<p>As recited in '652 Patent claim element 1(d), '497 Patent claim element 1(d), and '607 Patent claim element 1(f), Lo described measuring “e.g., SNPs” to determine percentage of foreign cfDNA in the sample, whereby “[t]he actual measurement could be done by real-time PCR, digital PCR, sequencing reactions (including massively parallel genomic sequencing) or any other quantitative methods.” B1220-B1290 (Lo U.S. 2009) at B1258, ¶ [0100]. In the Lo</p>

	exemplary fetal embodiment, “[t]he amount of maternal and fetal DNA in the plasma sample will be quantified, for example by the real-time PCR assays previously established ... or other types of quantifier well known to those of skill in the art,” citing references. B1220-B1290 (Lo U.S. 2009) at B1252, ¶ [0003].
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153. A POSA would understand the combination of conventional and commercially available laboratory techniques disclosed in Lo U.S. 2009 for detecting and quantifying different genotypes in a sample to be the same combination of laboratory techniques claimed in the Patents. The Patents do not claim any nonconventional use of the combination of laboratory techniques described in Lo U.S. 2009, nor do they claim any unique way of combining or using the techniques. Though, like Cantor 2007 discussed above, Lo U.S. 2009 often applies the combination of techniques for detecting fetal cfDNA in maternal blood, Lo also recognized its applicability to the analogous natural phenomenon of transplant cfDNA in a transplant recipient’s blood. B1220-B1290 (Lo U.S. 2009) at B1252, ¶ [0003]. Thus, like Cantor 2007, Lo U.S. 2009 confirms the conventionality and prior existence of the combination claimed in the Patents at least as of July 23, 2008 (the Lo U.S. 2009 filing date).

(c) Dhallan et al. – 2007

154. In another example, Dhallan *et al.* published a scientific paper in the journal Lancet at 369(9560):474-81 on February 10, 2007, B0428-B0435 (“Dhallan

2007”). Like the preceding Cantor 2007 and Lo U.S. 2009 disclosures, Dhallan 2007 establishes the conventionality of the Patents’ claimed combination for detecting multiple different genomes in a sample. The Dhallan 2007 authors note that “[t]he approach described here, which uses standard molecular biology equipment, allows for precise analysis of genetic material.” *Id.* at B0434.

155. Exemplary disclosures of Dhallan 2007 describing the combination of Patent claim elements are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	<p>As recited in ’652 Patent claim element 1(a), ’497 Patent claim element 1(c), and ’607 Patent claim element 1(a), Dhallan 2007 disclosed obtaining “formaldehyde-treated blood samples from [] pregnant women,” and using “free fetal DNA” from the samples “to diagnose fetal chromosomal abnormalities.” B0428-B0435 (Dhallan 2007) at B0428. The Dhallan “Methods” section states that plasma samples were prepared and cfDNA was extracted (as further recited in ’607 Patent claim element 1(b)) using standard, then-available commercial products:</p> <p style="padding-left: 40px;">Plasma and buffy coat samples were isolated in accordance with methods described previously. Genomic DNA was purified from both the plasma fraction and buffy coat of the same maternal blood sample, and the buffy coat fraction of the paternal sample, with the QIAamp DNA Blood Maxi Kit (Qiagen, Valencia, CA, USA). ... Plasma DNA was concentrated to 50-70 uL with a 10 kDa nominal molecular weight cutoff filter (Millipore, Bedford, MA, USA).</p>

	B0428-B0435 (Dhallan 2007) at B0429 (internal citations omitted).
Genotyping to obtain a SNP profile	Dhallan further described using the genotyping steps recited in '652 Patent claim element 1(b) and '497 Patent claim elements 1(a) and (b) (reciting genotyping to obtain polymorphism or SNP profiles, respectively), and '607 Patent claim element 1(c) (reciting selective amplification of at least 1,000 SNPs by PCR). Specifically, Dhallan disclosed that "SNPs were amplified from genomic DNA isolated from the maternal plasma ... by PCR," <i>id.</i> at B0429-B0430, further noting that "the human genome project has identified over 3.7 million SNPs to date," making "[e]ven on the smallest human chromosome ... about 54,000 genotyped SNPs [] available for analysis." <i>Id.</i> at B0434. Dhalla further disclosed genotyping homozygous and heterozygous SNPs, exemplifying that "[a]t certain SNP sites, the maternal genome will be homozygous for a nucleotide ... while at the same site the paternal genome might be homozygous for a different nucleotide" such that "the fetal genome will be heterozygous [] at the SNP site." <i>Id.</i> at B0428.
Sequencing	Dhallan described performing sequencing using "sequencing gels." <i>Id.</i> at B0430. Though '652 Patent claim element 1(c), '497 Patent claim element 1(d), and '607 Patent claim element 1(d) recite performing sequencing by multiplex or high-throughput sequencing, one skilled in the art as of the 2009 filing date of the Patents would have understood that the same sequencing performed in Dhallan 2007 using sequencing gels could have been accomplished using any of the numerous commercial multiplex/high-throughput sequencers available at the time (<i>see</i> section VII.B.3. above). In my own experience, as confirmed by the literature, it was standard and routine as of 2009 to use the same type of sample preparation, cfDNA extraction, and genotyping/selective amplification techniques disclosed in Dhallan in combination with one of the many commercial multiplex/high-throughput sequencing instruments to generate sequencing results for quantification as further recited in the Patent claims. <i>See</i> section VII.B.2. above.

Quantitating the foreign cfDNA	As recited in '652 Patent claim element 1(d), '497 Patent claim element 1(d) and '607 Patent claim element 1(f), Dhallan disclosed quantifying foreign cfDNA in the sample, stating that “us[ing] standard molecular biology equipment ... [f]ree fetal DNA is directly quantified from the heterogeneous mixture of maternal and fetal DNA in the maternal plasma.” <i>Id.</i> at B0434.
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156. Thus, a POSA would understand from the Dhallan 2007 disclosure, just like from the other literature cited above, that by 2007, “standard” laboratory techniques were available and used to accomplish the techniques claimed in the Patents.

(d) Beck et al. – 2009

157. In a further example, Beck *et al.* published a paper in the journal Clinical Chemistry at 55(4):730-738 in 2009 (B0436-B0444) (“Beck 2009”). Like the above-referenced literature, Beck 2009 demonstrates the conventionality of the Patents’ claimed combination for detecting multiple genotypes in a sample. In the case of Beck 2009, the authors used the combination of techniques to detect both pathogens (*e.g.*, viruses) and mutated cfDNA in otherwise apparently healthy human subjects. Beck 2009 describes an overview of the methods used therein, stating: “Serum DNA from 51 apparently healthy humans was extracted, amplified, sequenced via pyrosequencing (454 Life Sciences/Roche Diagnostics), and

categorized by (a) origin (human vs xenogeneic),²⁰ (b) functionality (repeats, genes, coding or noncoding), and (c) chromosomal localization.” *Id.* at B0436, Abstract Methods.

158. Exemplary disclosures of Beck 2009 describing the methods claimed in the Patents are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	As recited in ’652 Patent claim element 1(a), ’497 Patent claim element 1(c), and ’607 Patent claim elements 1(a) and (b), Beck 2009 explained that “[s]erum samples were collected and stored,” and that a commercial kit was used to extract the cell-free nucleic acids, wherein “[t]otal nucleic acids were extracted ... with the High Pure Viral Nucleic Acid Kit (Roche Applied Science) according to the manufacturer’s instructions.” B0436-B0444 (Beck 2009) at B0437. The authors further stated that “[w]e also collected EDTA-anticoagulated samples of whole blood from a subgroup of the volunteers (2 females, 2 males) and extracted genomic DNA with standard protocols.” <i>Id.</i>
Genotyping to obtain a SNP profile	Beck 2009 reported genotyping as recited in ’652 Patent claim element 1(b) and ’497 Patent claim elements 1(a) and (b) (reciting genotyping to obtain polymorphism or SNP profiles, respectively). Beck 2009 reported that “we have applied this high-throughput sequencing technology to generate an unbiased profile of the circulating DNA in healthy individuals.” B0436-B0444 (Beck 2009) at B0437. Beck 2009 described using “the BLAST program,” which is a publicly available database, as well as “a local install of the RepeatMasker software package (Institute for Systems Biology), which makes use of Repbase (version 12.09; Genetic Information Research Institute) and “querying

²⁰ A POSA would understand the term “xenogenic” to mean nucleic acids that originate outside the subject itself, and are from a foreign substance introduced into the subject, such as a virus or other pathogen in the context of this study.

	<p>databases of bacterial, viral, and fungal genomes, as well as the human genome (reference genome build 36.2),” including genomes “obtained from the National Center for Biotechnology Information (NCBI) (ftp://ftp.ncbi.nih.gov).” <i>Id.</i> These are all publicly available databases and software packages that include reference genomes for genotyping. The authors further compared the circulating cfDNA to genomic DNA in the subjects, looking for example, for polymorphic differences in single genes, repetitive elements, and foreign elements such as from non-human sources. <i>Id.</i> at B0438-B0439.</p>
Multiplex / high-throughput sequencing	<p>Beck 2009 described multiplex or high-throughput sequencing as described in ‘652 Patent claim element 1(c), ‘497 Patent claim element 1(d), and ‘607 Patent claim element 1(d), as well as sequencing by synthesis as recited in ‘607 Patent claim element 1(d). Specifically, Beck 2009 reported that “[t]he amplified DNA preparations were sequenced directly with a GS FLX genome sequencer (454 Life Sciences/Roche Diagnostics) according to the manufacturer’s instructions.” B0436-B0444 (Beck 2009) at B0437. As discussed in section VII.B.3. above, a POSA would understand the 454 sequencing instrument to employ sequencing-by-synthesis.</p>
Quantitating the foreign cfDNA	<p>As recited in ‘652 Patent claim element 1(d), ‘497 Patent claim element 1(d), and ‘607 Patent claim element 1(f), Beck 2009 disclosed quantifying foreign cfDNA in the sample, stating for example that “[t]o quantify the amounts of unidentified nucleotides, we counted and subtracted the masked nucleotides from the total nucleotide counts.” <i>Id.</i> at B0437. The authors further described determining whether “representation of the different chromosomes in the serum [cfDNA] is correlated with chromosome gene density or GC content,” which a POSA would understand to involve quantifying cfDNA to compare its “representation” or frequency in the cfDNA to that of genomic DNA. <i>Id.</i> at B0439. Beck 2009 also reported determining “representation of repetitive elements” in the cfDNA, which a POSA would</p>

	understand involves determining its relative fraction in the sample.
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159. Thus, a POSA would understand from the Beck 2009 disclosure, just like from the other literature cited above, that by 2009, the methods claimed in the Patents were routinely being used to detect multiple genotypes in a cfDNA sample.

(e) Beck et al. – 2010

160. In an additional example, Beck *et al.* published a scientific paper in the journal Molecular Cancer Research at 8(3):335-342, which was submitted to the journal on July 16, 2009 and published March 9, 2010 (“Beck 2010”) that further establishes the conventionality of the Patents’ claimed methods for detecting multiple genotypes in a sample, in this case in the context of cancer. B0445-B0453 (Beck 2010). I note that the submission date of July 16, 2009 indicates that the experiments underlying Beck 2010 were carried out before the Patents’ earliest filing date of November 6, 2009. Beck 2010 reported that “[c]irculating nucleic acids (CNA) isolated from serum or plasma are increasingly recognized as biomarkers for cancers,” and that related to the authors’ work, “[i]dentification of specific breast cancer-related CNA sequences provides the basis for the development of a serum-based routine laboratory test for breast cancer screening and monitoring.” B0445-B0453 (Beck 2010) at B0445, Abstract.

161. Exemplary disclosures of Beck 2010 describing the claimed methods are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	As recited in '652 Patent claim element 1(a), '497 Patent claim element 1(c), and '607 Patent claim elements 1(a) and (b), Beck 2010 reported that serum samples were collected from breast cancer patients, and after storage, using standard, then-available commercial products, "serum was centrifuged at 4,000 x g for 20 min and 200 uL of the supernatant was used in the High Pure Nucleic Acids Extraction Kit (Roche) according to the instructions of the manufacturer." <i>Id.</i> at B0446.
Genotyping to obtain a SNP profile	Beck 2010 further reported the genotyping steps recited in '652 Patent claim element 1(b) and '497 Patent claim elements 1(a) and (b) (reciting genotyping to obtain polymorphism or SNP profiles, respectively). Specifically, Beck 2010 reported that "[c]ancer-specific DNA perturbations such as microsatellite instability, mutations, sequence length, and promoter methylation patterns detected in serum/plasma have been proposed for the diagnosis and clinical assessment of cancer treatment." <i>Id.</i> at B0446. Beck 2010 further reported analyzing repetitive elements based on "assigned nucleotides." <i>Id.</i> ; <i>see also id.</i> at B0447. A POSA would understand that repetitive elements are a form of polymorphism that are prevalent in cancer.
Multiplex / high-throughput sequencing	Beck 2010 taught performing sequencing as recited in '652 Patent claim element 1(c), '497 Patent claim element 1(d), and '607 Patent claim element 1(d), which recite performing sequencing by multiplex or high-throughput sequencing, and by sequencing-by-synthesis, respectively. Specifically, Beck 2010 described sequencing "using a Roche/454 GS-FLX high-throughput sequencer," which as discussed in section VII.B.3. above, is a high-throughput sequencer that performs sequencing by synthesis.

Quantitating the foreign cfDNA	As recited in '652 Patent claim element 1(d), '497 Patent claim element 1(d), and '607 Patent claim element 1(f), Beck 2010 described quantitating the abnormal cfDNA, in that case cancer-specific cfDNA. Specifically, they stated their results as “[h]igh-throughput sequencing of total serum DNA shows differential representation of certain repetitive elements in the CAN of patients with breast cancer compared with healthy controls.” <i>Id.</i> at B0449. A POSA would understand that determining the “representation” of the analyzed polymorphisms involves quantifying them. A POSA would further understand that the statistical methods the authors used in Beck 2010 were standard and well-accepted means for quantifying data as of 2009.
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162. Thus, a POSA would understand from the Beck 2010 disclosure, just like from the other literature cited above, that by 2009, the methods claimed in the Patents were routinely being used to detect multiple genotypes in a cfDNA sample.

(f) Gordon et al. – 2009

163. In an additional example, Gordon *et al.* published a scientific paper in the journal Nucleic Acids Research at 37(2):550-556, which was published online December 5, 2008 and published in print in 2009. This paper also establishes the conventionality of the Patents’ claimed methods for detecting multiple genotypes in a sample. B0454-B0460 (“Gordon 2009”). The authors explained that “[n]ext-generation sequencing provided broad elucidation of sample CNAs [circulating nucleic acids],” further explaining that “we detected infection-specific sequences...” in order to identify pathogens that cause chronic wasting disease in cattle and elk cfDNA. *Id.* at B0454, Abstract.

164. Exemplary disclosures of Gordon 2009 describing the claimed methods are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	As recited in '652 Patent claim element 1(a), '497 Patent claim element 1(c), and '607 Patent claim element 1(a), the Gordon 2009 authors explained that “[s]erum samples were obtained” from cattle and elk, and cfDNA was extracted using a commercial kit: “The WGA4 GenomePlex® Single Cell Whole Genome Amplification Kit (Sigma) was used for the nucleic-acid extraction ... according to the manufacturer’s protocol.” <i>Id.</i> at B0455.
Genotyping to obtain a SNP profile	Gordon 2009 also reported using the genotyping steps recited in '652 Patent claim element 1(b) and '497 Patent claim elements 1(a) and (b) (reciting genotyping to obtain polymorphism or SNP profiles, respectively), and '607 Patent claim element 1(c) (reciting selective amplification of at least 1,000 SNPs by PCR). Gordon 2009 explained that “[a] total of 657,431 elk sequences were elucidated, including 401,733 from animals post-infection” and that they were “searched against the public protein and EST datasets” and matched against “public databanks” to genotype the subjects. <i>Id.</i> at B0456. The authors also reported that “[a] total of 595,037 quality bovine sequences were elucidated, including 311,786 from animals post-infection,” which also were genotyped against public databases. <i>Id.</i> Gordon 2009 further described using selective amplification to amplify “3261 control-only motifs and 2896 motifs present in infected-only cows” using PCR under conditions a POSA would understand to be standard and conventional. <i>Id.</i> at B0455.
Multiplex / high-throughput sequencing	Gordon 2009 described using multiplex or high-throughput sequencing, including sequencing-by-synthesis, as recited in '652 Patent claim element 1(c), '497 Patent claim element 1(d), and '607 Patent claim element 1(d), respectively. Specifically, Gordon 2009 reported using “a Roche/454 genome sequencer (GS-FLx) according to the manufacturer’s

	instructions.” <i>Id.</i> at B0455. A POSA would understand the 454 sequencer to be a high-throughput sequencer that uses sequencing by synthesis, as explained in section VII.B.3. above.
Quantitating the foreign cfDNA	In addition, as recited in ’652 Patent claim element 1(d), ’497 Patent claim element 1(d), and ’607 Patent claim element 1(f), Gordon 2009 reported that the data generated in the study may be used for “targeted characterization and quantification of disease-specific DNAs.” <i>Id.</i> at B0458. The authors further reported that “[t]he information from the elk study demonstrates that a time course analysis of the blood CNAs can greatly improve our ability to determine the diagnostic signals present” in such diseases. <i>Id.</i> at B0459. Accordingly, a POSA would understand that the combination reported in Gordon 2009 can readily include quantifying foreign cfDNA in the sample.

165. Thus, a POSA would understand from the Gordon 2009 disclosure, just like from the other literature cited above, that by 2009, the methods claimed in the Patents were routinely being used to detect multiple genotypes in a cfDNA sample.

(g) Holt et al. – 2009

166. Holt *et al.* published a scientific paper in the journal Bioinformatics, at 25(16):2074-2075, with advanced access publication on June 3, 2009. B0461-B0462 (“Holt 2009”). Holt 2009 also establishes the conventionality of the Patents’ methods for detecting multiple genotypes in a sample. The authors explained that they “present a method for estimating the frequencies of SNP alleles present within pooled samples of DNA using high-throughput short-read sequencing.” *Id.* at

B0461, Abstract. They further note that “[t]he method was implemented in Perl and relies on the open source software Maq for read mapping and SNP calling.” *Id.*

167. Exemplary disclosures of Holt 2009 describing the claimed methods are included in the table below:

Claim Element	Exemplary Disclosure
Obtaining a sample	'652 Patent claim element 1(a), '497 Patent claim element 1(c), and '607 Patent claim element 1(a) recite obtaining a sample of cell-free nucleic acids, and '607 Patent claim element 1(b) further recites extracting cfDNA from the sample. Holt 2009 analyzed a pool of DNA from six strains of the bacteria <i>Salmonella Patapyphi</i> A. <i>Id.</i> at B0461, Abstract. Though Holt 2009 did not use cfDNA obtained from blood as a sample, a POSA would appreciate that the same further combination of elements could be equally applied to the DNA extracted from a pool of bacteria as to the DNA extracted from a blood sample containing cfDNA.
Genotyping to obtain a SNP profile	Holt 2009 described using the genotyping steps recited in '652 Patent claim element 1(b) and '497 Patent claim elements 1(a) and (b) (reciting genotyping to obtain polymorphism or SNP profiles, respectively). Specifically, Holt 2009 explained that “[t]his approach facilitates genome-wide SNP detection among closely related isolates.” <i>Id.</i> at B0461. For this study, Holt 2009 analyzed “a set of 403 SNPs” to generate profiles of allele frequencies that differed as between the strains in the pooled sample. <i>Id.</i> at B0461-B0462. The authors stated that their method “relies on opensource software Maq for read mapping and SNP calling,” and that it is “freely available from ftp://ftp.sanger.ac.uk/pub/pathogens/pools/ .” <i>Id.</i> at B0461, Abstract. A POSA would understand this software for genotyping was standard and openly available as of 2009.
Multiplex / high-	As recited in '652 Patent claim element 1(c), '497 Patent claim element 1(d), and '607 Patent claim element 1(d), which recite multiplex or high throughput sequencing, and sequencing by

throughput sequencing	synthesis, respectively, Holt 2009 disclosed “us[ing] the Illumina GAI to sequence pools of DNA.” <i>Id.</i> at B0461. As discussed above in section VII.B.3., the Illumina Genome Analyzer (Illumina GAI) is based on Solexa high throughput sequencing technology that uses sequencing by synthesis.
Quantitating the foreign cfDNA	As recited in ’652 Patent claim element 1(d), ’497 Patent claim element 1(d), and ’607 Patent claim element 1(f), Holt 2009 reported that the data generated in the study was used to quantify the SNPs being analyzed. Specifically, Holt 2009 described determining “[t]he frequency of each SNP [] in [the pools] ... using information (read from Maq’s pileup output) on each read [] of [] reads mapped to the SNP locus...” <i>Id.</i> at B0461. The authors described using standard technology available through the open software, wherein “[f]requencies were calculated according to the following formulae, implemented in a Perl script which calls Maq to do the initial read mapping and SNP calling...” <i>Id.</i>

168. Thus, a POSA would understand from the Holt 2009 disclosure, just like from the other literature cited above, that by 2009, conventional laboratory techniques were available for obtaining a sample, genotyping, sequencing, and quantifying different genotypes in the sample.

D. The Dependent Claims Also Recite Well-Understood, Routine and Conventional Steps

169. None of the dependent claims of the Patents add a nonconventional application or inventive concept to the claims. In my opinion, each dependent claim recites well-understood, routine and conventional steps to observe a natural phenomenon. All of the dependent claims of the Patents depend from, and

incorporate by reference the limitations of claim 1 of the respective patents. I address the dependent claims, grouped by related claim elements, in turn below.

1. Dependent Claims Reciting Different Types Or Numbers Of Polymorphisms

170. Regarding the '652 Patent, dependent claim 2 recites different types of genetic polymorphisms of which the polymorphism profile of claim 1 may be comprised. Specifically, claim 2 recites use of a polymorphism profile that “comprises one or more genetic variations selected from single nucleotide polymorphisms (SNPs), variable number of tandem repeats (VNTRs), hypervariable regions, minisatellites, dinucleotide repeats, trinucleotide repeats, tetranucleotide repeats, simple sequence repeats, insertion elements, insertions, repeats, or deletions.” Dependent claim 11 recites wherein the polymorphism profile of claim 1 comprises at least one SNP.

171. Regarding the '497 Patent, dependent claim 6 recites “at least ten different homozygous or heterozygous SNPs are detected.” Dependent claim 17 recites that “the SNP profile comprises informative homozygous and heterozygous SNPs.” Dependent claim 25 recites the homozygous or heterozygous SNP comprises a marker having at least two alleles, each occurring at a frequency greater than 1% of the population.”

172. Regarding the '607 Patent, dependent claim 2 recites the “at least 1,000 [SNPs] comprise [SNPs] that occur at an allele frequency greater than 1% of a

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population. Dependent claim 3 recites the “at least 1,000 [SNPs] comprise homozygous [SNPs].” Dependent claim 4 recites the “selective amplification of target [DNA] sequences amplifies a plurality of genomic regions comprising at least 5,000 [SNPs].” Dependent claim 5 recites the “selective amplification of target [DNA] sequences amplifies a plurality of genomic regions comprising at least 10,000 [SNPs].”

173. As explained above in section VII.B.2., genotyping to obtain a polymorphism profile comprising SNPs was well-understood, routine and conventional as of November 2009. As explained in section VII.B.4., it was also well-understood, routine, and conventional as of November 2009 to genotype homozygous and heterozygous SNPs, and SNPs that arise from various mutations including insertions, repeats, or deletions. In addition, the written description makes clear that it was conventional to genotype SNPs having alleles that each occur at a frequency greater than 1% of a population. B0001-B0024 ('652 Patent) at B0015, 11:22-31. Moreover, as explained above in section VII.C.1., it was conventional, including using commercially available kits and products, to genotype 5,000 SNPs, 10,000 SNPs, and many more. Accordingly, there is nothing nonconventional or innovative in the limitations recited in '652 Patent claims 2 and 11; '497 Patent claims 6, 17, 18, 24, and 25; '607 Patent claims 2, 3, 4, and 5.

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2. Dependent Claims Reciting Different Types Of cfDNA

174. Regarding the '652 Patent, dependent claim 3 recites wherein the cell-free nucleic acids of claim 1 are DNA.

175. Regarding the '497 Patent, dependent claim 12 recites “the donor-specific circulating cell-free nucleic acids ... in the biological sample are DNA, RNA, mRNA, miRNA, double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA hairpins, or a combination thereof.”

176. It was routine and conventional as of Nov. 2009 to assay these recited types of nucleic acids using the claimed methods. The Patents' written description, again, states only at a high level of generality that “[n]ucleic acids from samples that can be analyzed by the methods herein include: double-stranded DNA, single-stranded DNA, single-stranded DNA hairpins, DNA/RNA hybrids, RNA (e.g. mRNA or miRNA) and RNA hairpins.” B0001-B0024 ('652 Patent) at B0014, 10:45-51; *see id.* at B0016, 13:17-21. The Patents do not disclose or claim any nonconventional laboratory techniques for analyzing any of these types of nucleic acids.

177. Neither the Patents' written description nor their claims indicate any nonconventional approach to assaying these particular types of nucleic acids, or any innovation based on analyzing any particular form of nucleic acid over another.

Accordingly, there is nothing nonconventional or innovative in the limitations recited in '652 Patent claim 3 and '497 Patent claim 12.

3. Dependent Claims Reciting Certain Common Modifications, Error Rates, Or Quality Scores Associated With Multiplexed Or High Throughput Sequencing

178. Regarding the '652 Patent, dependent claims 4 and 6 recite that the multiplexed sequencing of claim 1 is shotgun sequencing (claim 4) and sequencing at least ten different nucleic acids (claim 6).

179. Regarding the '497 Patent, dependent claim 3 recites a “high-throughput sequencing assay that generates at least 1,000 sequence reads per hour.” Dependent claim 4 recites “a high-throughput sequencing assay” that comprises “a next-generation sequencing assay.” Dependent claim 5 recites “the high-throughput sequencing assay generates sequencing reads of at least 36 bases.” Dependent claim 19 recites that the “sensitivity of the method is greater than 56%.” Dependent claim 21 recites a sequencing error rate of “less than 1.5%.” Dependent claim 23 recites using a “quality score.”

180. '607 Patent dependent claim 6 recites the “high throughput sequencing reaction comprises assigning a quality score to bases of said provided sequences.”

181. As explained above in section VII.B.3., and is also evident from the literature and product-related publications, all of these limitations are either themselves conventional methods, or are features inherent in the standard use of the

conventional methods recited in the independent claims from which they depend. For example, shotgun sequencing and next-generation sequencing are types of multiplex/high throughput sequencing that were well understood, routine and conventional as of Nov. 2009. In addition, high-throughput sequencing to generate at least 1,000 reads per hour ('497 Patent claim 3) and read lengths at least 36 bases long ('497 Patent claim 5) was inherent in all high-throughput sequencers available as of November 6, 2009, as demonstrated for example by Margulies *et al.*, which published that the instrument which became branded as the Illumina Genome Analyzer “is able to sequence 25 million bases ... in one four-hour run,” and generates an “[a]verage read length (bases)” of “108.” B1305-1309 (Margulies 2005) at B1305 (Abstract); B1307, Table 1.

182. Sequencing at least ten different nucleic acids using multiplex sequencing also was a standard application of the commercial multiplex sequencers at the time. For example, product literature from 2007 for the Illumina Genome Analyzer system states that an “excess of 2.6 billion bases of high-quality filtered data per paired-end run on a single flow cell (as of October, 2007)” could be achieved” (B0463-B0466) (Illumina 2007) at B0463 and the Illumina Genome Analyzer product literature from 2008 states that multiplexed sequencing of 96 different samples on a single flow cell could be achieved using their instrument. B0467-B0470, (Illumina 2008) at B0467. Generation of at least 1,000 sequence

reads per hour, and reads of at least 36 bases, also was a standard feature of the conventional operation of commercial high throughput sequencers in 2009. The Patents' written description states only at a high level of generality, in the "Sequencing" section generally discussing the multiple sequencing instrumentalities available in the market at the time, that:

In some cases, high throughput sequencing generates at least 1,000, at least 5,000, at least 10,000, at least 20,000, at least 30,000, at least 40,000, at least 50,000, at least 100,000, or at least 500,000 sequence reads per hour, with each read being at least 50, at least 60, at least 70, at least 80, at least 90, at least 100, at least 120, or at least 150 bases per read.

B0001-B0024 ('652 Patent) at B0017, 15:12-18. The Patents do not disclose or claim any nonconventional use of the multiplex/high-throughput sequencing methods elsewhere (including in that section) described in the Patents. A POSA in 2009 would understand this is an inherent feature in the standard use of the conventional multiplex/high-throughput sequencing machines referred to in the Patents.

183. The sequencing error rates and sensitivities also are inherent features of conventional use of the sequencing equipment, as discussed in section VII.B.3.c above.

184. Using a quality score likewise was conventional practice when using the standard high throughput sequencers available in 2009. B0467-B0470 (Illumina 2008) at B0467, B0469; B0471-B0480 (Li 2009b) at B0473. Moreover, I note that

the Patents' written description does not include any disclosure of nonconventional use of a quality score; rather, the only mention of "quality score" in the written description generically states that "[t]he use of quality scores for improved filtering of SNP calls, or the use of resequencing, should reduce error rate and increase sensitivity." B0001-B0024 ('652 Patent) at B0022, 26:51-53. The prior literature further described software that had already been developed and made commercially available before November 2009 to provide quality scores as recited in the Patent claims. *See, e.g.*, B0226-B0243 (Voelkerding 2009) at B0235 ("Image parameters such as intensity, background, and noise are then used in a platform-dependant algorithm to generate read sequences and error probability-related quality scores for each base. Although many researchers use the base calls generated by the platform-specific data-pipeline software, alternative base-calling programs that use more advanced software and statistical techniques have been developed.").

185. Neither the Patents' written description nor the claims identify anything nonconventional or innovative about the limitations recited in these dependent claims. To the contrary, they are routine applications, or inherent features, of the multiplex / high throughput sequencers when used in conventional ways, including in the 2009 timeframe. Accordingly, there is nothing nonconventional or innovative in the limitations recited in '652 Patent claims 4 and 6; '497 Patent claims 3, 4, 5, 19, 21, and 23; and '607 Patent claim 6.

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4. Dependent Claims Reciting Organ or Species Limitations

186. Regarding the '652 Patent, dependent claims 14 and 15 recite the transplant is selected from the group consisting of kidney, pancreas, liver, heart, lung, intestine, pancreas after kidney, and simultaneous pancreas-kidney (claim 14); or is heart or kidney (claim 15), respectively.

187. Regarding the '497 Patent, dependent claim 9 recites wherein the transplant recipient is human.

188. Each of these additional steps was well-understood, routine and conventional as of Nov. 2009, particularly when used in conjunction with monitoring of transplant status or rejection. The Patents' written description does not purport to attribute invention of any type of organ transplantation to the named inventors. Nor do the inventors contend they came up with the notion of analyzing the bodily fluid samples of human transplant recipients as opposed to those of some other species. *See* B0001-B0024 ('652 Patent) at B0012, 5:36-40. Accordingly, there is nothing nonconventional or innovative in the limitations recited in '652 Patent claims 7, 8, 9, 14, and 15; and '497 Patent claims 7, 8, 9, and 29.

5. Dependent Claims Reciting Conventional PCR Or Amplification Methods

189. Regarding the '497 Patent, dependent claim 10 recites that "an amplification reaction is performed on the donor-specific circulating cell-free

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nucleic acids in the biological sample prior to determining the amount of donor-specific circulating cell-free nucleic acids ... in the biological sample.”

190. As explained in section VII.B.2. above, performing an amplification reaction on donor cfDNA prior to quantifying it also was routine and conventional as of November 2009. Neither the Patents’ written description nor the claims identify anything nonconventional or innovative about the limitation recited in this dependent claim. To the contrary, it was a standard application that was routinely used in the 2009 timeframe. Accordingly, there is nothing nonconventional or innovative in the limitation recited in ’497 Patent claim 10.

6. Dependent Claims Reciting Different Sample Types

191. Regarding the ’652 Patent, dependent claim 12 recites that the sample of claim 1 is blood or serum.

192. Regarding the ’497 Patent, dependent claims 2 and 27 recite that the sample of claim 1 is blood (claim 2) or plasma (claim 27), respectively.

193. As explained above in section VII.B.1., obtaining a biological sample from a patient, including from blood, plasma, or serum, and including for use with the methods claimed in the Patent claims, was well-understood, routine and conventional as of Nov. 2009. Neither the Patents’ written description nor the claims identify anything nonconventional or innovative about the limitations recited in these dependent claims. To the contrary, they are standard applications that were

routinely used in the 2009 timeframe. Accordingly, there is nothing nonconventional or innovative in the limitations recited in '652 Patent claim 12 and '497 Patent claims 2 and 27.

7. Dependent Claims Reciting Further Genotyping Prior To Quantifying Cell-Free Nucleic Acids

194. '497 Patent dependent claim 15 recites “further comprising genotyping [donor, recipient, or both donor and recipient] prior to the determining in step (d).” The written description and claims do not disclose any unconventional application of further genotyping prior to determining an amount of donor-specific cell-free nucleic acids in the biological sample (as recited in claim step 1(d)), or any way in which the prior genotyping improves upon the recited conventional methods. The only relevant disclosure in the written description states that “[i]n some embodiments, both the donor and recipient will be genotyped prior to transplantation.” B0001-B0024 ('652 Patent) at B0013, 8:55-56; *see also id.* at B0016, 13:2-3 (same). This, however, is only a high-level generalization that does not provide any improvement over the routine and conventional techniques as recited in independent claim 1 from which the dependent claim depends.

8. Dependent Claims Reciting Certain Concentrations Of Cell-Free Nucleic Acids In The Sample

195. Regarding the '497 Patent, dependent claim 20 recites “the determining comprises detecting donor-specific circulating cell-free nucleic acids from the solid

organ transplant wherein the donor-specific circulating cell-free nucleic acids from the solid organ transplant make up between 0.03% and 8.0% of the total circulating cell-free nucleic acids in the biological sample.”

196. Regarding the ‘607 Patent, dependent claims 7 and 8 recite the “quantified amount of said kidney transplant-derived [cfDNA] ...” comprises certain percentages “of said total circulating [cfDNA]” in the samples, which are “between 1.5% and 8%” (claim 7) and “at least 0.05%” (claim 8), respectively.

197. As explained in section VII.B.4. above, the ability to detect any of these concentrations of cell-free nucleic acids in a sample is an inherent feature of the standard and conventional methods and instruments recited in the Patent claims. Neither the Patents’ written description nor claims identify any nonconventional or innovative use of these conventional techniques that renders them able to detect or quantify cfDNA at the recited concentrations when they would not otherwise be able to do so. Accordingly, there is nothing nonconventional or innovative in the limitations recited in ’497 Patent claim 20 and ’607 Patent claims 7, 8, 9, and 10.

E. Concluding Opinions Regarding Conventionality of the Recited Combination of Laboratory Techniques

198. In my opinion, based on my review of the Patents and understanding of the state of the art at the time the Patents were filed, the claimed methods of the Patents employ routine and conventional techniques already in use to detect the natural phenomenon of transplant cfDNA. The same techniques recited in the

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Patents were already in use for detecting fetal, cancer, and pathogen cfDNA, and those POSAs using the techniques for those applications recognized they would be equally applied in the transplant space.

199. The Patents' claims do not purport to improve upon or describe any nonconventional use of the recited existing combination of laboratory techniques. From the perspective of a POA in the 2009 timeframe, the combination of laboratory techniques recited in the Patents was well understood, routine and conventional, and already in use to detect a range of natural phenomena.

VIII. THE PATENT METHODS DO NOT CREATE ANY NEW PREPARATION

200. As explained above, the Standard Patents are directed to the steps of (1) obtaining biological samples containing cell-free nucleic acids from a transplant recipient; (2) genotyping the transplant donor and/or recipient to establish profiles of genetic polymorphisms (or SNPs)²¹; (3) performing multiplex or high-throughput sequencing of the cell-free nucleic acids to detect the genotyped polymorphisms (or SNPs); and (4) quantifying the transplant (donor-derived) cell-free nucleic acids in the sample using the genetic differences in the sequences. The method claims are not directed to a novel method of preparing a new composition. The claimed method steps do nothing more than observe that cell-free DNA is present in a transplant

²¹ The '607 Patent further recites selectively amplifying at least 1000 SNPs by PCR.

patient, and correlate the presence of the cell-free DNA with transplant rejection, using a conventional combination of techniques.

201. The claims recite the step of obtaining a biological sample containing cell-free nucleic acids from a transplant recipient. This step does not result in a novel composition, as it merely involves obtaining a naturally-occurring sample from a patient.

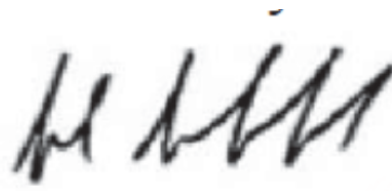
202. The claims also recite steps of genotyping the transplant donor and/or recipient to establish profiles of genetic polymorphisms and performing multiplex or high-throughput sequencing of the cell-free nucleic acids to detect the genotyped polymorphisms (or SNPs). The '607 Patent claims further recite selectively amplifying at least 1,000 SNPs by PCR in order to detect them. These steps use well-understood, routine and conventional—indeed commercially available—techniques for analyzing the naturally-occurring sample. They do not change the relative amounts of the transplant donor-specific and transplant recipient cfDNA being quantified in the claimed method.

203. Finally, the claims require quantifying the transplant (donor-specific) cell-free nucleic acids in the sample using the genetic differences in the sequences. This step also does not result in a new composition or preparation. As explained above, this quantification step is broadly recited with no further specificity and involves standard techniques for quantifying nucleic acids. This step also does not

change the relative amounts of the transplant donor-specific and transplant recipient cfDNA being quantified in the claimed method.

* * *

I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.



Dated: June 11, 2020

Professor John Quackenbush

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EXHIBIT 1

Genomic Tools and Labs

Oncologist Survey: MRD in Early Stages of Adoption

CONCLUSION

We surveyed 41 Oncologists including 38 who practice medical oncology and their views on minimal residual disease (MRD) testing. Overall, MRD has good penetration and is increasing. In addition, most ordering physicians surveyed already believe the test should be in NCCN guidelines. Guideline inclusion could represent a meaningful lift to ASPs. Over the next decade, we think MRD will emerge as one of the largest testing categories in oncology. While we think there will be several companies with hundreds of millions of revenue, our top pick in the space is Natera who we think will have a disproportionate share of our estimated industry revenue north of \$1B by 2027.

- **Docs are increasing the amount of MRD testing.** We asked two different ways how physicians are looking at MRD and in both ways, physicians said they are increasing their testing. This shouldn't be too surprising given where we're at in the adoption curve. That said, we're pleased the data confirmed what we should know: More physicians will order MRD and physicians already ordering MRD will likely order more tests.
- **Docs favor tumor informed for now.** 9/14 docs who use MRD said they believe tumor informed MRD is the more accurate test. We believe that MRD tests which don't need a tumor block as a reference will eventually replace tumor informed, but we think this would take new generations in assays and AI improvements. We think the next five years will be the era of tumor informed MRD. We expect the market could do \$1B in revenue by 2027. Tumor independent has the opportunity for patients when tissue is unavailable.
- **Docs see a path for guidelines.** To our surprise, fully half of the docs who have ordered MRD, think the test should be in guidelines. We noted, we asked about guidelines generally and not CRC. This is positive to us because CRC is where most of the compelling data is. It could imply physicians intuitively believe if it works in one tissue type, it should theoretically work in another.
- **Calls on MRD.** Natera has an underappreciated first mover advantage by virtue of its already having processed more than an estimated 100K bespoke assays. We believe all of those patients will continue their monitoring using Natera's assay. In addition, Natera has around a half decade of clinical trial data.
- **Not so small niches.** Even in the near term, tumor independent has the opportunity to be used in around 1/4th of cases. That 1/4 represents those cases in which a sufficient tumor sample is unavailable, and thus tumor informed isn't an option. We think companies such as Exact could pair the assay successfully with prognostic testing. Lastly, companies such as Neogenomics and Quest could help bring these assays to the community setting in a meaningful way.

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Related Companies:	Share Price:
DGX	123.48
EXAS	66.85
GH	25.93
NEO	12.15
NTRA	45.82
NVTA	0.67
ADPT	5.07

INDUSTRY RISKS

Competition, regulation, COVID-19 related uncertainty, early stage of commercialization of some testing modalities

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Oncologist Survey – MRD in Early Stages Of Adoption

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The Differentiated Diagnostic – Piper Sandler’s Proprietary Checks in Diagnostics

Piper Sandler’s Difference – The “Differentiated Diagnostic” is Piper Sandler’s proprietary assessment of markets, lab services, and products in the genomic tools and lab space. We provide a unique perspective, though finding non-traditional resources and conducting primary research outside the conventional sellside thinking so we, AND YOU, can make better investment decisions. If it’s not Piper’s Differentiated Diagnostic, it’s just the same old consensus checks.

Part 1

The Takeaways

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Stock Calls

Our focus call:

- **Natera is our top pick given the highest exposure to minimal residual disease.** We model Signatera to have more than \$400MM in revenue by 2024. We believe Natera's competitive position is solidifying by taking an overwhelming lead in the space, via high early adoption rates. We believe after 2024, Natera will have many competitors but it will have a first mover advantage.
 - We think the first mover advantage helps benefit Natera because it will be the first to longitudinal data at longer increments. In other words, by being the first mover, Natera will have the first data sets with 30 months of patient outcomes, 36 months, 42 months, etc.
 - We think, particularly in community oncology, ordering particular tests becomes habitual. We think Natera has the ability to retain high numbers of early customers and that Natera can at least grow with market rates for MRD, which we see growing potentially in the high double digits to triple digits for a half decade.

Survey Response Summary in MRD

We estimate at least 30% of oncs are now doing MRD

- 14/38 (37%) of respondents noted that they had used Signatera in the past 12 months, and 6/38 (16%) said they had used Guardant's Reveal test within the past 12 months.

Of the 14 oncologists who have used tumor informed MRD (i.e. Signatera) within the past 12 months, we saw higher levels of utilization of tumor informed MRD. The respondents are also increasing their MRD usage, and view Signatera as their preferred test, with it having the characteristics they want in a MRD test. Many also think it has sufficient evidence for NCCN guideline inclusion.

- 6/14 noted that they are increasing ordering of NGS/tumor informed MRD, 8/14 said they were ordering about the same amount, and none said they were decreasing ordering
- 9/14 said they are increasing their amount of MRD testing generally, with 1/14 disagreeing
- 9/14 agreed that Signatera is their preferred MRD test, with 2/14 disagreeing
- 9/14 agreed that tumor informed MRD has better performance characteristics vs. tumor agnostic approaches with 1/14 disagreeing
- 7/14 agree that there is sufficient evidence for tumor informed MRD to be in NCCN guidelines, with 2/14 disagreeing

Unfortunately, MRD has a ways to go in terms of utility studies. It's perceived as having varying levels of utility, in different settings:

- The adjuvant setting: The most utility (5/14 rating it as having strong/very strong utility, 3/14 rating it as having little or no utility)
- The surveillance setting: A more mixed view of its utility (4/14 rating it as having strong/very strong utility, 4/14 rating it as having little or no utility)
- The neoadjuvant setting: The least utility (2/14 rating it as having strong/very strong utility, 5/14 rating it as having little or no utility)

Piper's Multiyear Vision of the MRD Market

MRD will likely end up having grown in the high double digits in 2023, and even still, we believe it is widely underpenetrated (we estimate low single digit penetration). We recommend investors have a high degree of exposure to this rapidly growing marketing, which is already in excess of \$250M on an annualized basis. We think industry revenue will exceed \$1B by 2027.

We highlight what we see as important trends:

- We think Natera's first mover advantage in tumor informed MRD will help them sustain market leading growth. As an important note, our oncologists checks find that a majority believe tumor informed is superior to tumor independent in terms of specificity. Given the longitudinal use of that assay, the majority of oncologist we speak to believe specificity is more important than sensitivity.
- That said, tumor agnostic/naïve will likely be used frequently given the convenience and ability to access patients unable to be serviced by tumor informed (likely 25% of cases). We think Guardant is the best positioned company in that market.
- We note, over the very long term, we think tumor independent will be the predominant approach as sequencing costs become lower and advancements in AI improve the assays. We think there will no longer be meaningful gaps in performance by that time. That said, we think that will take 5-10 years time and it not yet in.
- We think market will be dominated by Natera but other players will find important niches in the market. That said, we think this market will be billions so these "niches" are nonetheless valuable. While it's tough to pick "winners" in the later to market tumor informed vendors, we think the companies that carve out expert areas might be best positioned. For example:
 - If Exact Sciences can combined Oncotype scoring with monitoring
 - NeoGenomics/Quest bringing the market to the community setting
 - Other smaller players having superior data or go to market strategies with certain tissue types.

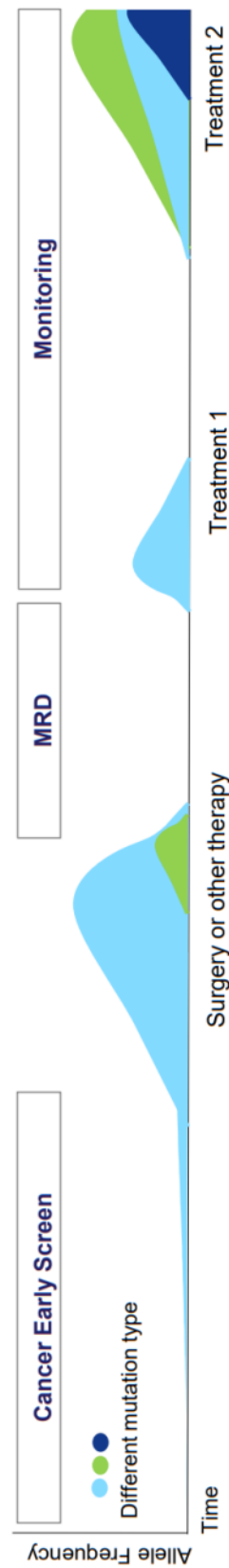
Part 2

MRD Testing in Solid Tumors Overview

Appx16148

MRD in Solid Tumor Cancers

- MRD means minimal residual disease or the amount of tumor DNA that's in the body. It's a number (or a yes or no answer depending on the test) that can be tracked serially in a patient who has been previously diagnosed with having cancer.
- There are two major categories of MRD in solid tumor: Tumor informed and tumor agnostic.
 - Tumor informed testing involves sequencing a sample from a cancer that has not been treated (generally an exome), with PCR testing afterwards to compare blood samples against the initial tumor profile to detect relapses
 - Tumor naïve (we interchange with tumor independent or tumor agnostic) testing refers to tests that measure the levels of circulating tumor DNA (ctDNA) in a patient's blood, with Guardant's being NGS based
- We estimate the number of tests has grew well over 100% to over 200K tests in 2022 from less than 80K in 2021. We estimate that Natera has about 90% market share today. Behind Natera, in solid tumor MRD is Guardant Health who we estimate ran between 10K and 20K tests last year. Companies such as Invitae and NeoGenomics mostly played a role in pharma services MRD.
- We estimate dozens of companies have MRD assays in development. We expect many of them to come to market in the next few years.
- For MRD in blood cancers, please see our [ADPT note here](#)



MRD TAM currently at \$35B and Expanding Rapidly

We peg the current solid tumor MRD TAM at ~\$35B (lumping together our estimates for the most readily addressable solid tumor cancers). We believe that through 2027, there is significant potential for additional indications to open up, and that testing frequency and the length of testing may increase significantly, potentially opening up a very large TAM (described below). In our view, the three most penetrated opportunities will likely be as follows:

- CRC: \$7.4B
- Bladder: \$4.2B
- Breast: \$14.0B
- IO Monitoring: \$9.9B

With slightly less than \$200M in revenue across all solid tumor indications in 2022 (our estimate), we believe that the market will expand through private reimbursement and new indications over the coming five years, with a CAGR in the mid to high double digits. More specifically, we estimate that by 2025-2027, the MRD TAM will reach >\$60B as more indications are covered, and that penetration will increase significantly. We believe that the market will be rapidly penetrated from 2025-2030, with revenues of ~\$1-\$2B by 2027. We also expect that in 2027, Natera will have a 60%+ market share in MRD vs. our estimate of >70% currently overall, and well over >90% in solid tumors.

We also forecast that substantial new markets will open, with only ~2.5M solid tumor patients currently having access to MRD (vs. 13M cancer survivors in the US for the mid term population of cancer survivors for whom indications are in pipelines (of a ~19M total in the country)). We estimate that solid tumors will account for the vast majority of testing, with blood based cancers accounting for <15% of all cancers.

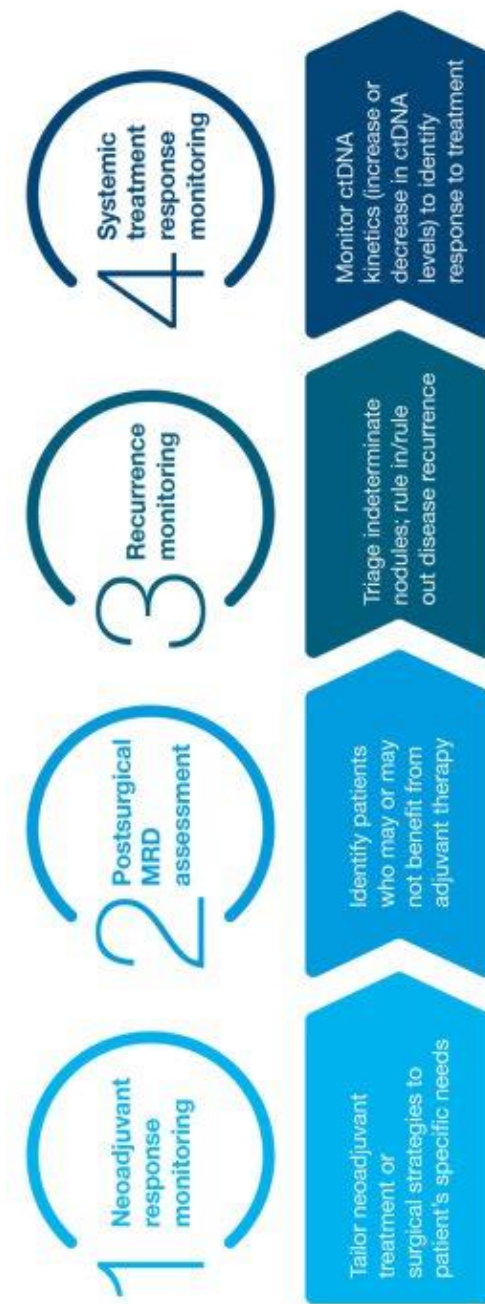
Cancer Type	Lower Estimate (in \$B)	High Estimate (in \$B)	Median (in \$B)	Estimated Medicare Coverage	Estimated Private Coverage	>50% Private Coverage	First to Coverage
Colorectal Cancer TAM	\$3.0	\$11.7	\$7.4	Covered	Covered	2024	Natera
Bladder Cancer TAM	\$1.7	\$6.6	\$4.2	Covered	Covered	2024	Natera
Breast Cancer TAM	\$5.7	\$22.3	\$14.0	Covered	Covered	2025	Natera
Ovarian Cancer TAM	\$0.4	\$1.7	\$1.1	2024	2024	2025	Natera
Esophageal Cancer TAM	\$0.4	\$1.5	\$1.0	2024	2024	2025	Natera
Lung & Bronchus Cancer TAM	\$4.7	\$18.5	\$11.6	2024	2024	2026	Natera
Pancreatic Cancer TAM	\$1.2	\$4.7	\$3.0	2024	2024	2026	Natera
Melanoma TAM	\$2.1	\$8.3	\$5.2	2024	2024	2026	Natera
IO Monitoring TAM	\$4.0	\$15.7	\$9.9	Covered	Covered	2025	Natera
Remaining Catch all Cancers	\$11.0	\$43.2	\$27.1	2027	2027	2028	All
Total	\$34.2	\$134.2	\$84.2				

Source: Piper Sandler

Natera - Signatera Introduction

Signatera is a blood MRD test used for cancer detection and surveillance. It is personalized for each patient. It is used for molecular residual disease assessment and for treatment response monitoring. The test launched in 2017. The test is a tumor informed test meaning an exome is initially ordered. The test finds the 16 most relevant mutations then tests serially for quantitative changes in this tumor.

As a reminder, Signatera, Natera's MRD test for solid tumors, is an industry leader in minimal residual disease testing, with data from >3,500 patients, and >15 peer reviewed publications, and 100+ clinical studies in the pipeline to drive coverage and guidelines. The test has seen rapid growth over the past few years, and in our view, will continue to be a rapid grower. In our view, this growth will be driven both by increased utilization by new doctors, in new cancer indications, as well as increased frequency of testing.



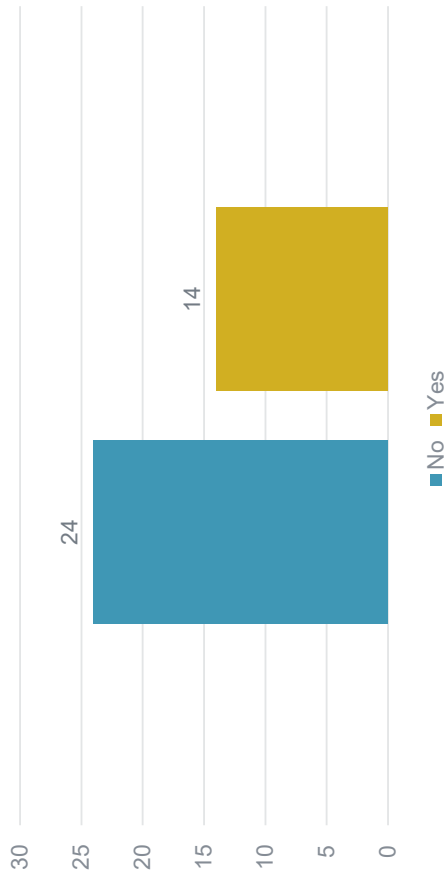
Source: Piper Sandler, Natera.com

Signatera has been ordered by 37% of Medical Oncs

Given the dominance of Signatera, we looked for solid tumor MRD penetration rates through asking about the brand name Signatera. We did this because we've experience checks with oncologists that have used profiling tests for monitoring purposes. In fact, for quality control purpose, we also asked the question "have you used NGS based tumor informed MRD?" and got N=14.

Consistent with Natera's estimate, our checks found 37% penetration (Natera quotes over 30%). We think penetration among academic oncologists is likely over 50% and community is likely under 30%. We think the biggest lever of growth over the next few years will be in penetration of community oncologists.

Have you used [Tumor informed MRD (brand name Signatera)] in the past 12 months?



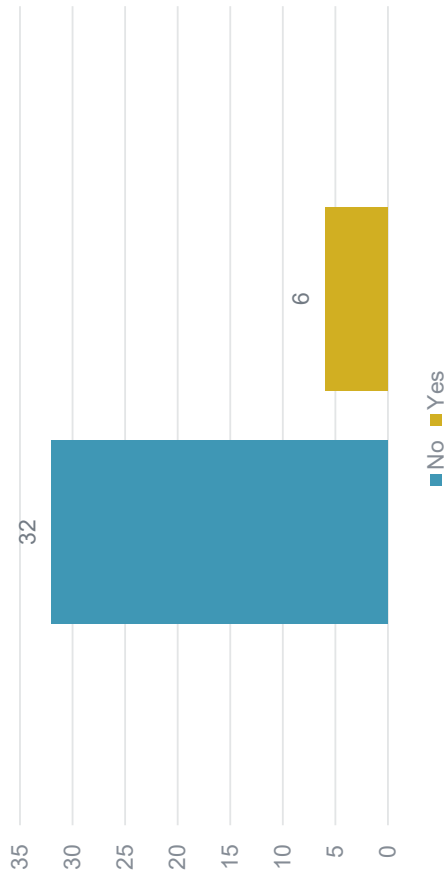
Source: Piper Sandler

And Reveal has been used by 16% of Medical Oncs

Guardant Reveal is an MRD test that doesn't require tissue. This provides meaningful convenience in the form of turnaround time. In addition, we've found that many times, the physician or pathologist cannot fully access the tissue (or doesn't have any to spare). In this particularly case (we estimate happens 1/4 of the time), tumor agnostics is the only option. We estimate Guardant is the clear leader in tumor agnostic MRD and few companies are going after this market. Behind, Guardant are Exact Sciences (who's first focus is tumor informed) and Grail (part of Illumina). Natera is also working on a tumor agnostic assay but likely doesn't discuss it much because of channel messaging.

To the surprise of many, Guardant360 has been used as an MRD for years. In fact, on our initial checks on looking for first adopters of Guardant's official MRD assay Reveal, we accidentally stumbled upon Guardant MRD users who have been using Guardant360 for years as their preferred MRD test. We think these users are low hanging fruit to adopt Guardant Health Reveal. Although we think Natera will still be the market share leader in MRD in the coming years, we think Guardant will build out a large use case.

Have you used [Tumor agnostic MRD (brand name Reveal)] in the past 12 months?



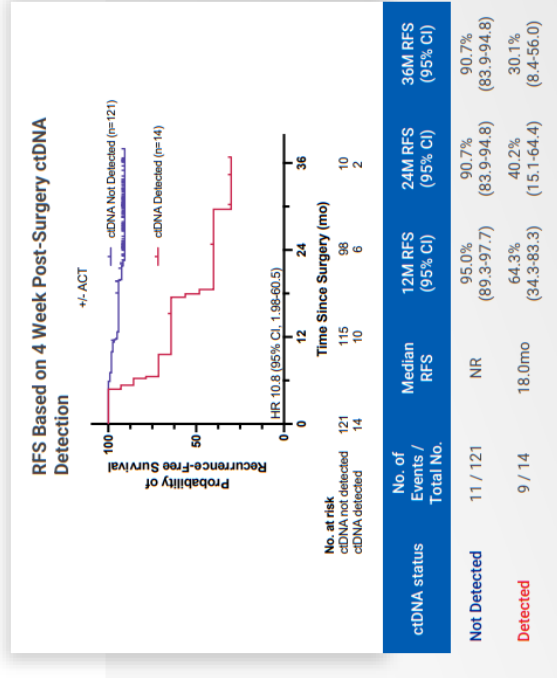
Source: Piper Sandler

Guardant Health – Tumor Agnostic has some improvement ahead

We note that the earliest iterations of tumor independent assays were slightly behind in terms of performance characteristics. That said the earliest Guardant Reveal was still able to achieve reimbursement for adjuvant monitoring. We note, tumor independent MRD is a much more data intensive and sequencing heavy process vs. tumor informed. Because of the difficulty, the performance characteristics tend to lag from a pure specificity standpoint. Saying that, it can also have sensitivity improvements beyond what a tumor informed assay can do. In fact, we think as sequencing becomes less expensive, the assay can lean on more data points to make a “call.” We suspect in 2024, Guardant should receive CMS coverage for recurrence in CRC and breast cancer. We note, during GH’s recent investors day presentation the company presented with 80% sensitivity and 99% specificity for recurrence. We think that will meet the CMS threshold for coverage.

COSMOS-Colon Update: Stage II/III completely resected (R0) population

INVESTOR | 20
DAY | 23



80%

Sensitivity
prior to or at recurrence

99%

Specificity
(sample-level)

Data to be included in Medicare CRC surveillance submission

Source: Piper Sandler, Guardant Investor Presentation

Other up and comers in MRD

We estimate there more than 20 companies actively pursuing MRD including most if not all of the major comprehensive genomic profiling companies and most of the liquid biopsy screening companies. For good reason, as we estimate the market will be the largest market in diagnostics. We think the MRD front runners are companies such as Natera, Guardant and Adaptive who have clear commercial traction. We think most of the other companies will need to rely on a product bundle or some form of differentiation that isn't price. On the tumor informed side, we find a lot of companies will use number of mutations tracked as a key differentiator. While we think there's a likely a good number, our research suggests that won't resonate with docs as differentiation. Another area of differentiation is level of detection. We think over time this will be a true differentiator. However, in the near term, as long as the protocol for oncologists is to go to CT scan, we think picking up even smaller levels of cancer cells won't matter as much as physicians will trust the CT scan over the ctDNA (something we think needs to change over time). In other words, having a technology that can enable the detection of cancer well before a confirmatory C-scan won't help until the studies prove it.

As the cost of sequencing decreases, we think most companies will move from an upfront exome to a whole genome. As far as we know, that would not constitute a new assay required for further clinical trials to support reimbursement. Several of the more significant tests currently under development include those by the following **covered** companies:

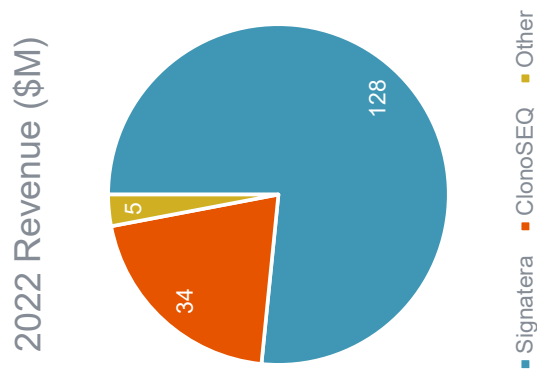
NeoGenomics – RaDaR is a tumor informed liquid biopsy test, with initial targets including cancers of the lung cancer, breast cancer, colon cancer and head and neck squamous cell carcinoma (HNSCC). RaDaR received its first commercial payer coverage in June 2023, for its pan-cancer testing option, by Blue Shield of California.

Exact Sciences – The company announced in August 2023 that it would be launching its MRD test in late 4Q23, with the brand name OncoDetect. The test is a tumor informed, ctDNA detection test. The company expects to apply for a LCD in mid/late 2024. The company noted that the MAESTRO technology, licensed from the Broad Institute, could potentially be applied to increase sensitivity for the test in the future and allow for additional mutations to be tested.

Invitae – The company received its first commercial coverage decision for its tumor informed test in March 2023, from Blue Shield of California. Invitae PCM is a pan-cancer, tumor-informed liquid biopsy assay co-developed with the TRACERx consortium that uses a next generation sequencing (NGS) to analyze ctDNA in a patient's plasma.

Haystack (Quest) – Acquired by Quest Diagnostics in April 2023 for \$300M, Haystack is being adapted by Quest, initially for clinical lab services available beginning in 2024. Development efforts will focus initially on MRD tests for colorectal, breast and lung cancers. Quest estimates the acquisition would be accretive by 2026.

Clinical MRD market share



The MRD market currently has few competitors, and we estimate that >95% of the current revenue in the space is comprised of ADPT and NTRA's tests. While several companies exist in the early stages, we believe they are primarily working to get additional volume, with the expectation of receiving better reimbursement in the future as additional payors adopt the tests. We note, the other in this case is predominantly Guardant.

We note, this graph mostly captures clinical MRD. Pharma services, particularly milestones and upfronts is not routinely disclosed by companies but is nonetheless meaningful. Companies such as Guardant and Neogenomics likely have meaningful MRD revenue in clinical development in excess of the \$5M shown on the graph above.

Source: Piper Sandler

Part 3

MRD Survey Data

Appx16157

Physician utilization – NGS/Tumor informed MRD

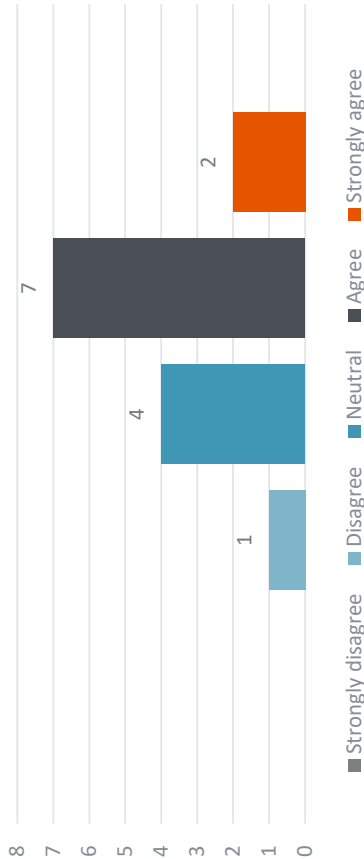
MRD is clearly in the market expansion phase. We think it's going to grow by both penetration of oncologists and increasing utilization of oncologists. We note that zero oncologists surveyed are decreasing their usage of tumor informed MRD. That said, we are early in the adoption cycle so that shouldn't come as a surprise. We expect tumor informed MRD to grow two ways. First by increasing in penetration rate of oncologists (37% having used it in the past 12 months in our survey) and increasing number of patients oncologists use the test on. We also note, we only used the 14 oncologists who answered they are doing MRD. As such, this question is subject to small sample size issues.

Those oncologists who use MRD testing are doing so at increasing rates, which we think over time could lead to a significant increase in the utilization of these tests. We think MRD represents potentially the highest volume testing modality in all of cancer diagnostics with the opportunity to test more than 4x per year per patient on the 18M cancer survivors in the US.

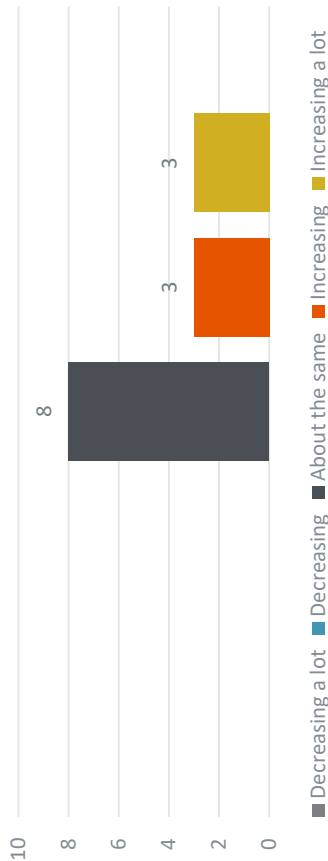
We estimate the more likely candidates are the 1.8M new cancer patients tested 4x per year but that still pegs the market at \$29B. We think that's an underestimate. For example, if your family members had been in remission even a long as 10 years ago, wouldn't you want them tested for recurrence? As of today, we estimate that less than 300,000 MRD tests (between Adaptive, Guardant, and Natera) have been ran in totality and the majority of those have not been reimbursed.

In all, we estimate that CMS has reimbursed ~\$350M to date in MRD testing, spaced out over the last 4 years. We estimate, albeit at a small base, that MRD testing is growing in the mid to high double digits quarter-on-quarter and will grow in the triple digits annually for at least a few years. Although our lofty TAM estimate assumes high compliance, we feel comfort in our view that MRD testing dwarfs profiling testing and with a reimbursement rate at a comparable level to a test that's done only a couple times; we think a \$3,920 price for MRD is unsustainable.

[Oncs who do tumor informed MRD, n=14] "I'm increasing my amount of MRD testing"



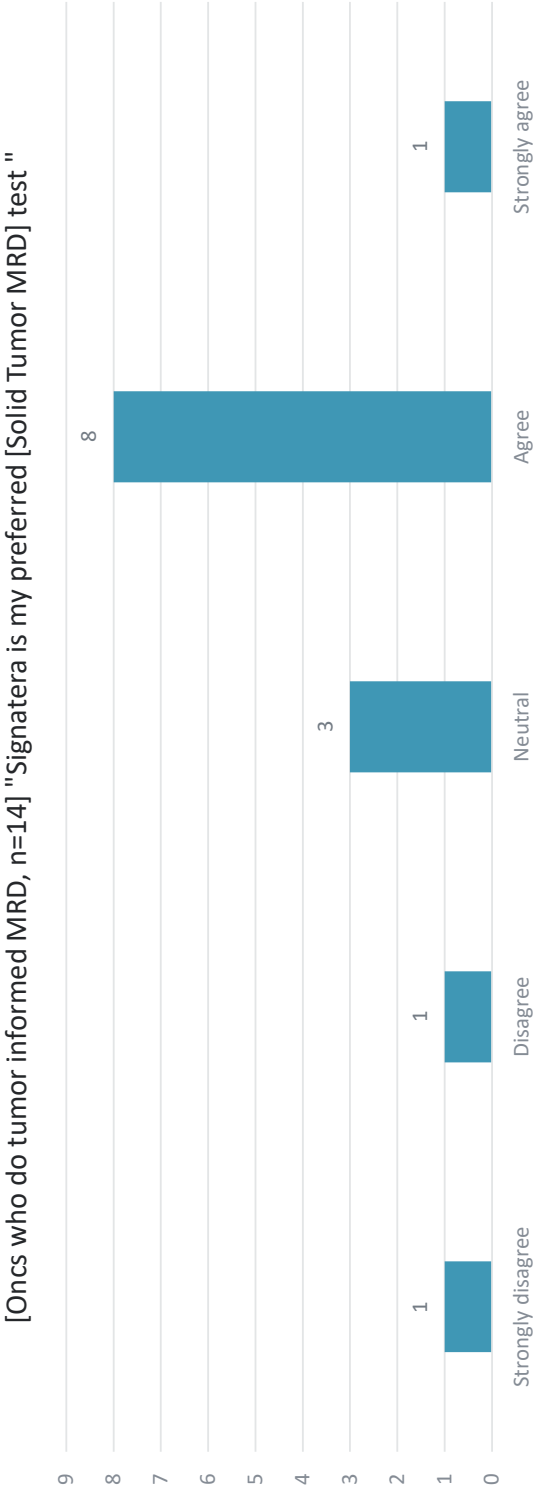
[Oncs who do tumor informed MRD, n=14] "In [NGS/tumor informed MRD], are you increasing or decreasing ordering?"



Signatera is widely seen as the preferred test

Among oncologists who use tumor informed MRD, there was a strong preference for Signatera, with 9/14 saying it was their preferred test. We would be interested to learn what other tests the two who disagreed use, and suspect they may either be using alternative technologies for recurrence detection such as imaging technologies, or the MRD technologies of Guardant (as a reminder 5/6 who use Reveal also use Signatera) or other up and coming companies in the clinical trial setting.

While we think Signatera is the preferred test is positive, we note that there's really nothing else on the market outside of clinical trials. We suspect, the two people that disagree, probably use one in that setting.



Source: Piper Sandler

Tumor informed vs. Tumor Independent (Naïve)

Tumor informed MRD testing utilizes cancer tissue samples to calibrate the test to that specific cancer and provide accurate results. While we notice that the broad consensus is that tumor informed tests perform higher, we think the gap will close over the next ~5-10 years. We point to clinical trials as a catalyst to the change the opinion of oncologists. Tumor independent testing, alternatively, looks for ctDNA or other markers in blood samples, while often times having sensitivities that rival tumor informed tests.

Many oncologists we've spoken with have noted the utility of tumor independent tests for patients who in whom cancers are difficult to access (about 10-20% of the time), or who have already received surgery, removing the initial tumor, such that an initial exome can no longer be used to inform future testing.

We think the reality is that both approaches are necessary. Over the next 3-5, we think the market will be 80% tumor informed and 20% tumor independent with the vast majority of the tumor independent tests being used in the tumor unavailable use case. Our reason for this breakdown is that tumor informed has seen such rapid adoption that we think it is winning out in the market. That said, there are so many convenience factors that will still pull volumes to tumor Independent that we think there will be ample opportunity for tumor independent companies to expand the pie and gain some share from tumor informed.

We've ran several checks with oncologists on the differences between tumor informed and tumor agnostic. For lack of a better description, we find that oncologists just find tumor informed more intuitive. The scientific difference in accuracy is likely debatable. A tumor informed assay is more likely to catch something it's specifically targeting and PCR in general has a higher accuracy than NGS. That said, tumor agnostics can catch mutations outside of the predetermined panel.

Tumor informed vs. Tumor Independent

	Key Differences	Select companies
Tumor informed	Potential for high sensitivity due to personalized variant tracking	Caris, Natera, Foundation, Invitae/Archer, Exact Sciences, Inivata (NeoGenomics), Personalis, C2i Genomics, Predicine
	Potential to apply personalized approach across tumor types (presumed independently of cancer specific optimization)	
	Strong synergies with tissue WES/CGP assay portfolios	
	Likely well positioned for heterogeneous cancers where a generic panel may not sufficiently cover inter patient variability	
Tumor independent	Faster turnaround time for initial blood result (particularly well suited for late stage monitoring an early stage cases where adjuvant treatment may begin within 4 weeks)	Guardant, Grail, Caris, Predicine
	Convenient sampling logistics, which are better suited for centralized and decentralized testing alike.	
	Epigenomic signatures expected to increase technical performance without need for tissue.	
	Likely well positioned for homogenous cancers where a set of common mutations are applicable across patients and cancers where tissue is limited even in early stages	

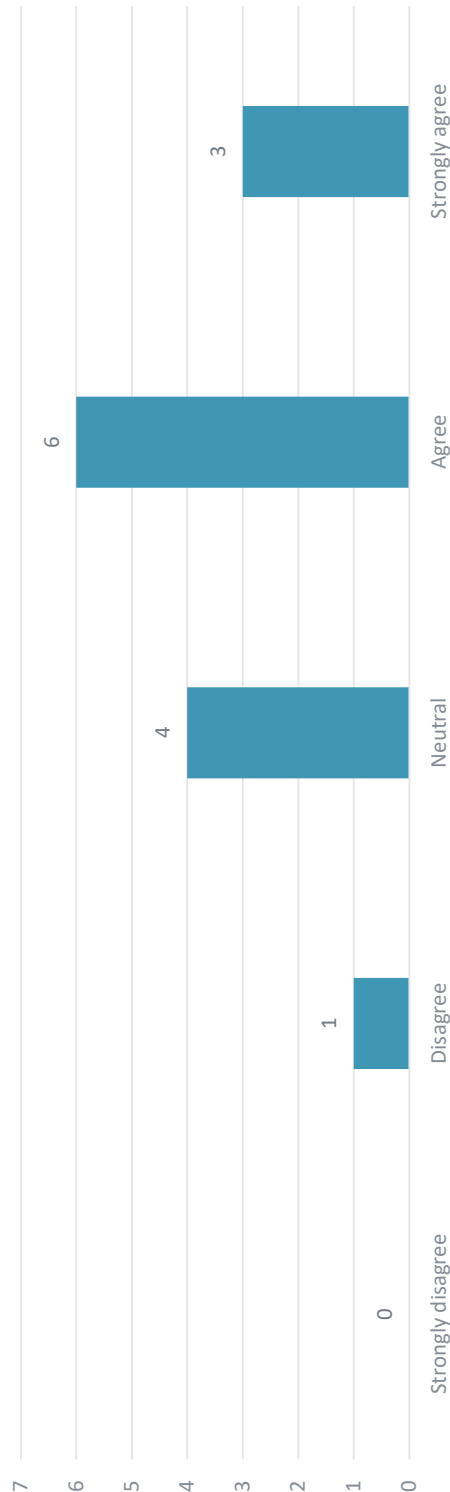
Source: DeciBio

Tumor informed testing seen as superior to tumor independent

We don't necessarily think tumor informed MRD is actually significantly more accurate than tumor independent. In some circumstances, for example, when a patient develops a brand new mutation, tumor informed can be less accurate. That said, our interviews with oncologists have universally found that tumor informed is more intuitive and has the perception of higher accuracy. This survey widely confirmed that bias.

Our interview also seem to confirm the quantitative nature of the tumor informed readout gives confidence to the findings of the reports they received. In other words, seeing a report that says the patient has 300 mutations in the blood feels more accurate than just a report that says "positive for minimal residual disease." Lastly, there are some technological difference. While both assays use NGS on the first read, tumor informed uses PCR on the serial testing portion of the business. PCR tends to have almost perfect accuracy. Over the very long term (a decade from now), as sequencing costs get lower and sequencing depth increases multiple fold, we think tumor independent testing will eventually make up the majority of tests. We note, our survey was in late July/Early August, prior to the early termination of Cobra.

[Oncs who use tumor informed MRD, n=14] "Performance of tumor informed MRD is higher than tumor agnostic"

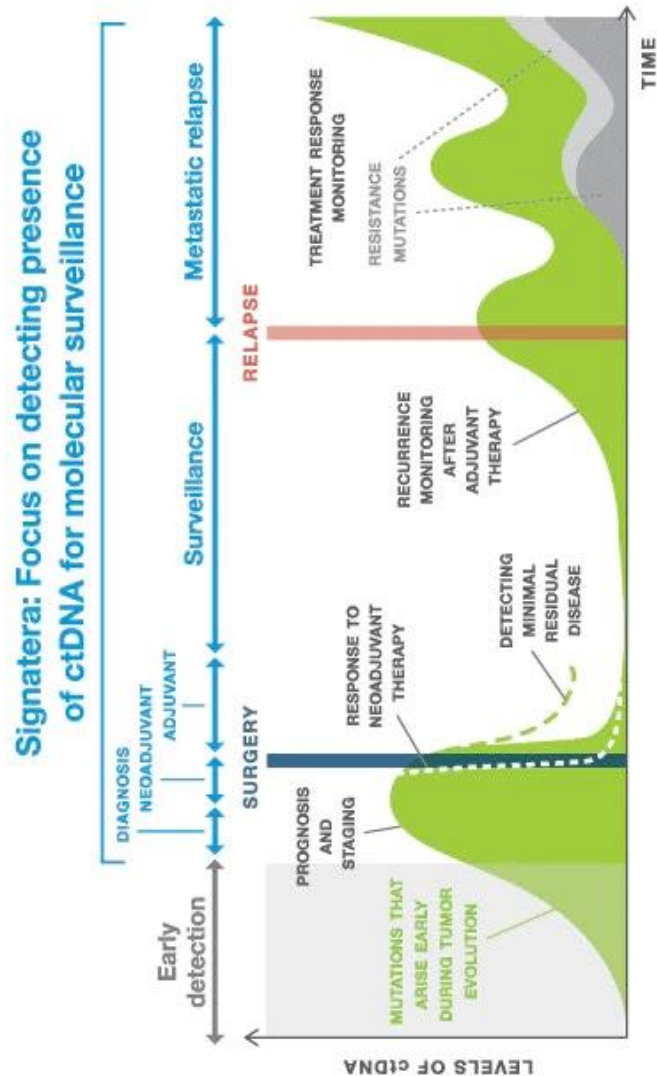


Source: Piper Sandler

Neoadjuvant vs. Adjuvant and Monitoring

The neoadjuvant refers to the setting prior to surgery, when chemotherapies are used with the intention of shrinking tumors, or stopping their spread. Adjuvant settings refer to those after surgeries, with the intention of killing any remaining cancerous cells. Monitoring refers to the setting after the adjuvant setting, in which testing occurs to determine whether there have been relapses of cancer in the patient.

Many cancer types release small levels of cancer markers that provide doctors and researchers insight into the presence of the cancer and effectiveness of the treatment. The levels and patterns of these markers can correlate with the location, stage, and severity of the cancer. Doctors can order an MRD test to monitor the treatment course, confirm or monitor remissions, find recurrence sooner, identify patients who have a higher risk of relapse, and inform doctors on when to restart treatment. The image below shows why MRD testing is done. The figure shows the scenario emphasizing the importance of MRD detection after the initial treatment. When MRD detection is not performed, there is no indication of how effective the treatment was on the tumor, and relapse may eventually occur. If MRD diagnosis confirms a positive result, the patient can be prescribed a more personalized treatment to prevent any future relapses. Ultimately, the most important benefit of MRD will be improving outcomes. As MRD sees higher penetration rates, we expect companies such as Natera to run studies showing the results of performing intervention or lack of intervention because of MRD, and whether MRD leads to better outcomes.

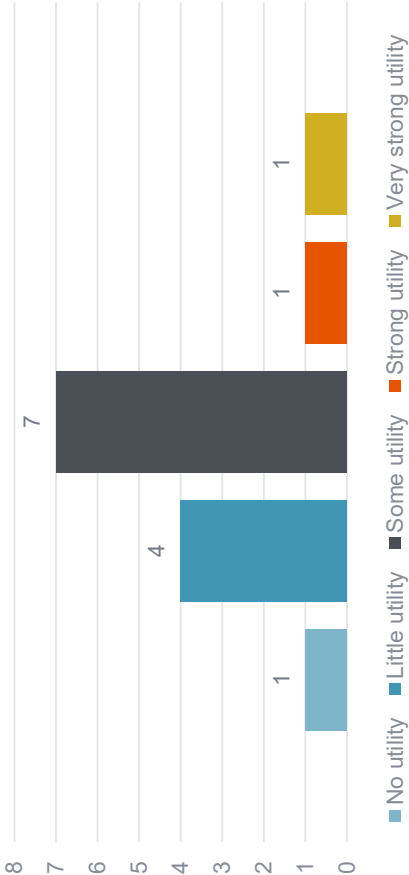


Source: Piper Sandler
NTRA presentation

Less utility seen for MRD in the neoadjuvant setting

The respondents said that the neoadjuvant setting is the setting in which the least value can be derived from MRD. In this setting, drugs are typically used to shrink or eliminate tumors. However, most cancer treatments would still require additional testing including imaging to determine whether tumors are still existent rather than just having shrunk sufficiently that they are shedding little DNA; MRD testing also would likely not negate the use of surgery in many cases.

[Oncs who do tumor informed MRD, n=14] How much utility do you see for MRD testing in [the neoadjuvant setting]?



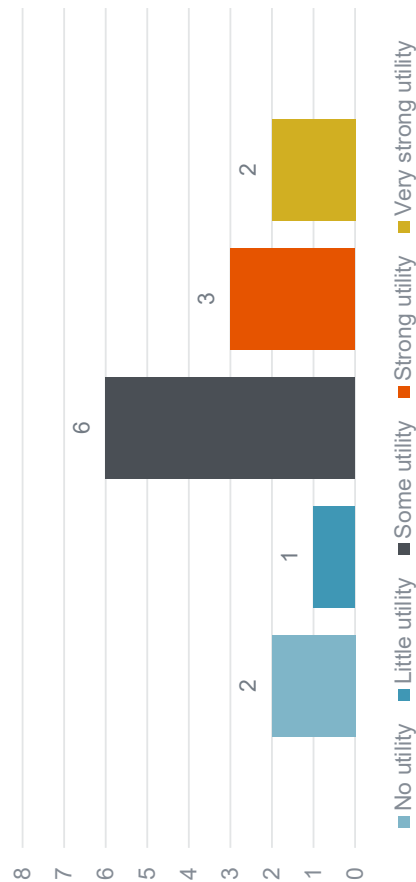
Source: Piper Sandler

Greatest utility for MRD seen in the adjuvant setting

We saw more agreement on the utility of MRD in the adjuvant setting than any other setting, with 5/14 agreeing that it provides value. As a reminder, MRD testing in this setting would show whether a cancer has successfully been treated by surgery. MRD testing begins after an interval post treatment so that residual cancer cells can be removed by the patient's body.

As a comp, Guardant was recently approved for testing in the adjuvant setting, with its initial ctDNA test 4-6 weeks post surgery (or 2-4 after completion of systematic therapy).

[Oncs who do tumor informed MRD, n=14] How much utility do you see for MRD testing in [the adjuvant setting]?



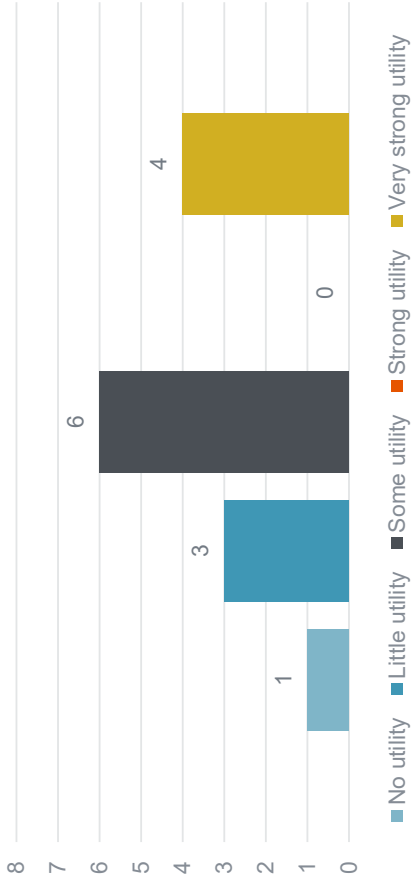
Source: Piper Sandler

Mixed opinion on MRD’s utility in surveillance

Equal numbers of respondents said that there is utility for MRD in monitoring, with 4 viewing it as providing utility and 4 thinking it had little or no utility. We think this may be a combination of the low levels of surveillance testing being performed.

As a comp, Guardant’s test was recently approved in the surveillance setting every 3-6 months after treatment for two years, and 6-12 months for the following three years. These tests are only now becoming an option, but even so, there are still likely questions as to whether or not testing once per 6-12 months would provide sufficient lead time prior to symptoms associated with a recurrence for a patient to get back on to a treatment plan.

[Oncs who do tumor informed MRD, n=14] How much utility do you see for MRD testing in [the surveillance setting]?



Source: Piper Sandler

What NCCN guidelines are and why it matters

National Comprehensive Cancer Network (NCCN) guidelines are used as industry standard recommendations by various private payers. A positive guideline inclusion of a type of test, or specific companies test, generally leads to many payers reimbursing for various tests.

We have heard comparisons between MRD testing and existing NCCN guidelines for conducting CT scans on patients who have been treated for solid tumors, for monitoring. One complaint about finding recurrence via CT scans is that there is scant evidence that the testing actually improves survival rates.

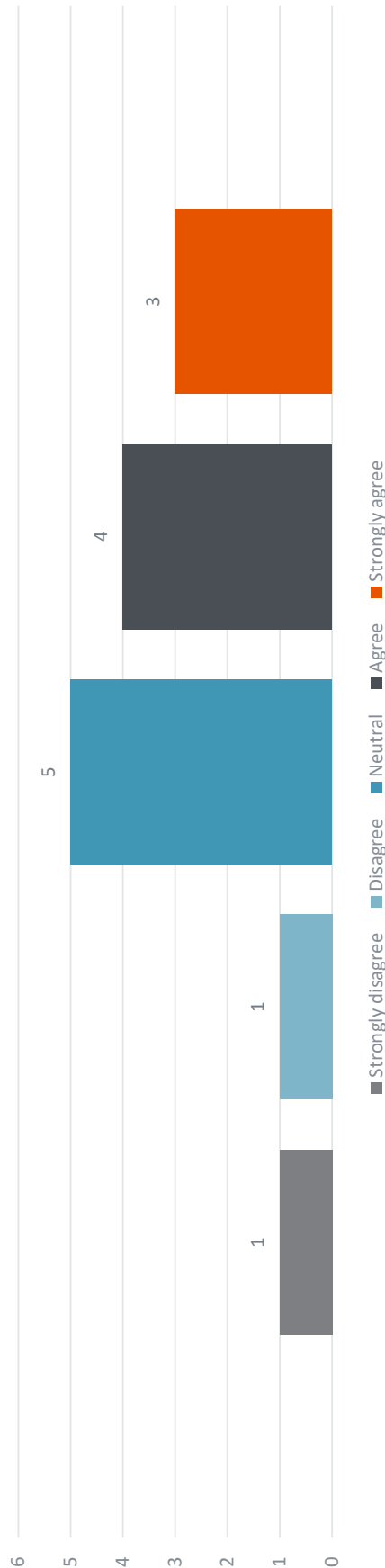
In our view, payors will want to see that the testing is improving survival rates in order to approve the testing – particularly for expensive testing, such as NGS. In fact, the recent price increase from Medicare to ~\$3,900 likely would decrease the frequency private payors would cover the test, assuming they follow Medicare's pricing decision.

That said, in Signatera's case, we think that the majority of NCCN committee members are generally for inclusion of Signatera, but that they want a bit more evidence on its utility before being instituting guideline inclusion.

Oncologists that use MRD, think it should be in Guidelines

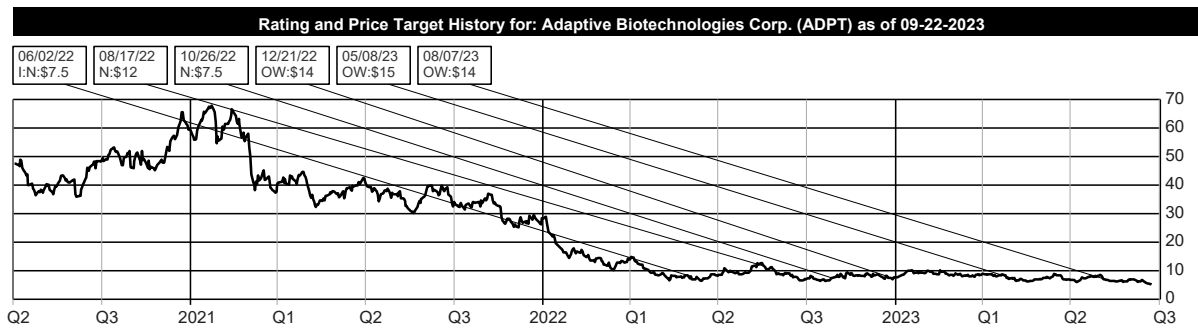
In our view, the half of respondents (7/14) who believe that MRD merits guideline inclusion is high, considering that the tests are still early in their commercialization and that new data is being released on them on a regular basis. We think that this view is positive for companies with these tests, and think that it will increase over time as the tests are more widely used and as they are seen as having greater utility.

[Oncs who do tumor informed MRD, n=14] "I think there's sufficient evidence for tumor informed MRD to be in NCCN guidelines"



Source: Piper Sandler

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R: Resuming Coverage
T: Transferring Coverage
D: Discontinuing Coverage
S: Suspending Coverage
OW: Overweight
N: Neutral
UW: Underweight
NA: Not Available
UR: Under Review

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HOLD [N]	377	37.29	50	13.26
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**IN THE UNITED STATES DISTRICT COURT
FOR THE MIDDLE DISTRICT OF NORTH CAROLINA**

NATERA, INC.,

Plaintiff,

v.

NEOGENOMICS LABORATORIES, INC.,

Defendant.

C.A. No. 1:23-cv-629

**CONTAINS HIGHLY CONFIDENTIAL
INFORMATION**

REPLY DECLARATION OF DR. MICHAEL METZKER

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I, Michael Metzker, declare as follows:

1. I previously submitted a declaration¹ in the above-captioned litigation with respect to the infringement of U.S. Patent Nos. 11,530,454² and 11,519,035³ (collectively, the “Natera Patents”). I incorporate that declaration herein by reference, including information regarding my qualifications and expertise, including my curriculum vitae, as well as my understanding of relevant legal principles.

2. I have been asked to consider the Declaration of NeoGenomics’ expert Dr. Brian Van Ness⁴ which I understand has been submitted in support of NeoGenomics’ opposition to Natera’s motion for a preliminary injunction regarding NeoGenomics’ RaDaR™ test. In the Van Ness Declaration, Dr. Van Ness has offered opinions regarding the invalidity and inventorship of the Natera Patents as well as opinions responding to my Opening Declaration. I have also considered his deposition testimony concerning the Van Ness Declaration (“Van Ness Tr.”).⁵ I respond and reply to his opinions herein.

¹ See Dkt 17, *Declaration of Dr. Michael L. Metzker in Support of Plaintiff Natera, Inc.’s Motion for a Preliminary Injunction*, dated July 29, 2023 (“Metzker Op. Decl.”).

² See Dkt. 1-1, NAT-NEO-00000001-00000222, U.S. Patent No. 11,530,454 to Babiarz *et al.*, (“Detecting mutations and ploidy in chromosomal segments”) (the “’454 Patent”).

³ See Dkt 1-2, NAT-NEO-00000223-00000435, U.S. Patent No. 11,519,035 to Rabinowitz *et al.*, (“Methods for simultaneous amplification of target loci”) (the “’035 Patent”).

⁴ See Dkt. 116, *Declaration of Dr. Brian Van Ness in Support of NeoGenomics Laboratories, Inc.’s Response in Opposition to Natera, Inc.’s Motion for a Preliminary Injunction*, dated October 18, 2023 (“Van Ness Declaration” or “Van Ness Decl.”).

⁵ See Ex. 1, Transcript of Deposition of Brian Van Ness, Ph.D., dated October 25, 2023 (“Van Ness Tr.”).

I. LEGAL STANDARDS

3. I am not an attorney and have no legal training. In this Reply Declaration, I have applied certain legal principles of which I have been informed by Natera’s counsel as described in my Opening Declaration⁶ and *infra*.

A. Section 101

4. In evaluating whether patent claims are patent-eligible under 35 U.S.C. § 101, I have been informed and understand that a Court conducts a first inquiry to determine whether the claims at issue are directed to a patent-ineligible concept. I understand that, if this threshold determination at step one is met, the Court moves to the second step of the inquiry and considers the elements of each claim both individually and as an ordered combination to determine whether the additional elements transform the nature of the claim into a patent-eligible application.

5. I have been informed and understand that the “directed to” inquiry in step one of the eligibility analysis considered in light of the specification is based on whether the claims as a whole directed to excluded subject matter. I understand, for example, new and improved techniques for producing tangible and useful results fall outside the categories of inventions that are “directed to” patent-ineligible concepts. Moreover, I understand that the “directed to” inquiry cannot simply ask whether the claims involve a patent-ineligible concept, because essentially every routinely patent-eligible claim

⁶ See Metzker Op. Decl. § III.

involving physical products and actions involves a law of nature and/or natural phenomenon.

6. I have been informed and understand that step two of the patent-eligibility analysis has been described as a search for an inventive concept. At step two, I understand that more is required than well-understood, routine, conventional activity already engaged in by the scientific community, which fails to transform the claim into significantly more than a patent upon the ineligible concept itself. I understand that, at this step, claims that are “directed to” a patent-ineligible concept yet also improve an existing technological process are sufficient to transform the process into an inventive application of the patent-ineligible concept.

B. Section 103

7. I have been informed and understand that a patent is invalid for obviousness if the differences between the subject matter sought to be patented and the prior art are such that the subject matter as a whole would have been obvious at the time the invention was made to one of ordinary skill in the art to which said subject matter pertains. I understand that obviousness is a question of law based on underlying findings of fact. An analysis of obviousness must be based on several factual inquiries: (1) the scope and content of the prior art; (2) the differences between the prior art and the claims at issue; (3) the level of ordinary skill in the art at the time the invention was made; and (4) objective evidence of nonobviousness, if any. I am informed that a party seeking to invalidate a patent as obvious generally must demonstrate by clear and convincing evidence that a skilled artisan would

have had reason to combine the teaching of the prior art references to achieve the claimed invention, and that the skilled artisan would have had a reasonable expectation of success from doing so.

8. I have been informed and understand that where the prior art gives only general guidance as to the particular form of the claimed invention or how to achieve it, relying on an obvious-to-try theory to support an obviousness finding is impermissible. Moreover, I understand that obviousness cannot be shown “by merely throwing metaphorical darts at a board” in hopes of arriving at a successful result when the prior art gives either no indication of which parameters are critical or no direction as to which of many possible choices is likely to be successful. Rather, I understand that courts should reject such “hindsight” claims of obviousness.

9. Moreover, I have been informed and understand that evidence of secondary considerations of non-obviousness must always be considered when present, for example, to guard against hindsight bias. I am informed that such evidence of secondary considerations may often be the “most probative and cogent” evidence in the record to establish that an invention appearing to have been obvious was not.

C. Section 112 – Written Description

10. I have been informed and understand that the test for sufficiency of a patent’s written description is whether the disclosure of the application relied upon reasonably conveys to those skilled in the art that the inventor had possession of the claimed subject matter as of the filing date. I understand that this test requires an objective inquiry into the

four corners of the specification from the perspective of a person of ordinary skill in the art.

11. I have been informed and understand, based on that inquiry, the specification must describe an invention understandable to that skilled artisan and show that the inventor actually invented the invention claimed. I understand, however, that the description requirement does not demand any particular form of disclosure, and that the specification does not need to recite the claimed invention in the same words as recited in the claims.

II. TECHNICAL BACKGROUND

12. I identify certain technical principles that inform my opinions set forth in this Reply Declaration *infra*. In particular, I explain the challenges that one of ordinary skill in the art would have faced in working with cell-free DNA (cfDNA) that circulates in the body. Both Dr. Van Ness and I discuss cfDNA. In my view, the analysis in the Van Ness Declaration⁷ does not adequately describe the significant difficulties and challenges that faced one of ordinary skill in the art with respect to the use and manipulation of cfDNA to achieve the methods claimed in the Natera Patents, particularly in view of conflicting testimony Dr. Van Ness gave in his deposition. I also note Dr. Van Ness's admission that he has never published any work involving cfDNA and that his laboratory spent only about six months attempting to work with cfDNA in the early 2000s, and did not work on cfDNA

⁷ See Van Ness Decl. at ¶¶ 43-46.

again after the early 2000s. Nor has Dr. Van Ness published any work on tumor-specific genetic variants using cfDNA.

Q. You never worked on – you, yourself, in your lab never worked on cell-free DNA after the early 2000s, correct?

A. We never – after the early 2000s, we did not work on cell-free DNA, although all the methods of DNA extraction, multiple, and sequencing were routine in my lab. But we did not work on specifically cell-free DNA after the early 2000s.⁸

Q. Okay. I'll ask the question again. You and your lab have never published any work describing experiments using cell-free DNA, correct?

A. I have not. Only blood DNA we've worked on, but I have not looked at cell-free. We did not publish on cell-free.⁹

Q. You never published any work on tumor-specific SNPs or single nucleotide variants in cell-free DNA, correct?

A. We did publish on SNPs in tumor – unique to tumors. It wasn't from the cell-free DNA.¹⁰

A. Cell-Free DNA and Plasma Samples

13. Prior to 2014-2015, and even earlier, there were many obstacles to the assaying and analysis of cfDNA. These challenges were, and still are, greater than when working with DNA from tissue samples, such as samples of tumors. I discuss some of these challenges in this section. Dr. Van Ness agrees, as he testified in his deposition.

⁸ See Van Ness Tr. at 246:21-247:4 (objection omitted).

⁹ *Id.* at 247:15-22 (objection omitted).

¹⁰ *Id.* at 249:11-15.

Q. You and your lab were not consistently able to detect cell-free DNA with good sensitivity, correct?

A. In the early 2000s, we were all having some problems in the consistency of detecting cell-free DNA.

Q. And in fact –

A. Detecting the target of cell-free DNA we were looking for.¹¹

14. For example, Volik (2016)¹² provides a helpful summary of certain problems and difficulties facing scientists working with cfDNA. These challenges are also acknowledged in certain prior art references cited in the Van Ness Declaration, such as Forshew (2012).¹³ I discuss them *infra*.

15. As a general matter, cfDNA is difficult to biochemically process and analyze because it is relatively low-quality compared to DNA from other sources, such as tissue samples. In his deposition, Dr. Van Ness agreed that working with cfDNA posed technical challenges.

Q. Okay. Do you – you see here that Dr. Forshew, in that first sentence, says “Circulating DNA is fragmented to an average length of 140 to 170 base pairs and is present in only a few thousand amplifiable copies per milliliter of blood, of which only a fraction may be diagnostically

¹¹ *Id.* at 248:10-20 (objection omitted).

¹² See Ex. 2, Volik *et al.*, *Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies*, 14 *Mol. Cancer Res.* 898 (2016) (“Volik (2016)”).

¹³ See Dkt. 1-16, NAT-NEO-00043262-00043275, Forshew *et al.*, *Noninvasive identification and monitoring of cancer mutations by targeted deep sequencing of plasma DNA*, *SCIENCE TRANSLATIONAL MEDICINE* 4:136ra68, pp. 1-12 (2012) (“Forshew (2012)”).

relevant”? Do you disagree with Dr. Forshew’s statement?

A. I don’t.

Q. In the second sentence, a few lines down, it says, “But also the challenges involved in analysis of circulating tumor DNA, where mutated loci and AFs may be more variable.” Do you disagree with him that there are challenges involved in analysis of circulating tumor DNA because of those variable mutated loci and AFs?

A. He’s referring to past challenges that involve the analysis of circulating tumor DNA. *I agree with that.*¹⁴

16. Here, I provide an example of one such problem. Consider a cfDNA molecule that contains a desired single nucleotide polymorphism (“SNP”), which is a genomic variant at a single base position in DNA. If the cfDNA is fragmented such that the cfDNA fragment does not contain sites for both primers of a primer pair to bind that primer pair will not be able to amplify that particular SNP successfully when PCR is performed.

17. As of 2014-2015, scientists were beginning to explore how to use cancer-associated cfDNA.

Known to be present in the blood of cancer patients for decades, cell-free DNA (cfDNA) is beginning to inform on tumor genetics, tumor burden, and mechanisms of progression and drug resistance.¹⁵

¹⁴ See Van Ness Tr. at 116:19-117:21 (emphasis added).

¹⁵ See Volik (2016) at Abstract.

18. Indeed, the art recognized that blood plasma contained a highly variable content of cfDNA. In the case of cancer patients, for example, the level of tumor-associated cfDNA (known as “circulating tumor DNA” or “ctDNA”) would vary, including relative to the normal cfDNA that represents the majority of cfDNA found in plasma.

The amount of cfDNA in cancer patients varies widely.¹⁶

Not unexpectedly, in addition to varying absolute levels of cfDNA, the fraction of DNA molecules in the circulation of cancer patients that can be recognized as being derived from tumor cells also varies.¹⁷

19. The art recognized that the fragmented nature of cfDNA made it difficult to manipulate through existing techniques such as PCR-based amplification and sequencing. Dr. Van Ness agrees that there are challenges in amplifying cfDNA, as he testified in his deposition.

Q. Is it your opinion that there are particular challenges in amplifying cell-free or plasma DNA that one doesn't encounter in amplifying genomic DNA from tissue?

A. ***There are some challenges.*** And there are some challenges in whole genomic DNA amplification as well. Amplification was well known in the art, and, yes, there are challenges.¹⁸

Dr. Van Ness further acknowledged that “[t]here are challenges to amplifying genomic DNA. ***There are challenges to amplifying cell-free DNA.*** And are they the same? No,

¹⁶ *Id.* at 890.

¹⁷ *Id.*

¹⁸ *See* Van Ness Tr. at 109:25-110:7 (emphasis added).

the size of the fragments are different. The number of cycles you might want to do with cell-free DNA may be different than if you have a lot of genomic DNA from the tumor.”¹⁹

20. In addition, tumor genomes are genetically unstable and prone to mutate. A frequent consequence of cancer-associated genetic instability is that different populations of cells within the same tumor from a single patient can have different genetic mutations. When these different tumor cell populations release ctDNA into the plasma, one population’s ctDNA can have different sequences from other populations’ ctDNA. These different mutations among different cancer cells in a tumor are known as “subclonal” mutations. Biochemically processing and analyzing ctDNA sequences is therefore typically more challenging than simply collecting and processing DNA directly from healthy tissues and tumor tissues for study.

The fragmentation, low yield, and variable admixture of normal DNA present formidable technical challenges for realization of this potential.²⁰

[The] low amount, high degradation, and high admixture of normal DNA in cfDNA pose major challenges for the development of sensitive and robust detection pipelines. The fact that most (if not all) tumors are characterized by multiple subclonal populations with only a subset of somatic mutations shared among all cells (for review, see ref. 52) further complicates the issue.²¹

¹⁹ *Id.* at 113:4-10 (emphasis added).

²⁰ *See* Volik (2016) at Abstract.

²¹ *Id.* at 901.

21. Moreover, ctDNA is typically low-abundance in the plasma of a person with cancer relative to the levels of other cfDNA in the plasma.²² Specifically, the plasma is likely to contain far more cfDNA from non-cancer cells than ctDNA from tumor cells, which makes preparing preparations of the ctDNA more difficult (because it must be distinguished in some way from the non-tumor cfDNA).

Circulating tumor DNA (ctDNA) is typically so diluted by normal DNA that existing sequencing methods (e.g., Sanger sequencing) were not sufficiently sensitive to detect mutant DNA molecules. As a result, mutation-specific PCR was the only available technology that could provide sufficient specificity for detection of the weak tumor signal.²³

However, *tumor-derived DNA usually constitutes only a small percentage of total cfDNA* so the ability to detect rare genome aberrations is an essential requirement for cfDNA analysis pipelines.²⁴

22. In deposition, Dr. Van Ness acknowledged particular and “unique” challenges associated with multiplex amplification of cfDNA in the context of discussing amplification of different types of cfDNA (e.g., cfDNA from patients who received an organ transplant, or pregnant women, or subjects with cancer).

Q. I was asking about multiplex amplification, the actual multiplex PCR reaction. That – do you – is there something you have to do differently in performing multiplex PCR to amplify transplant cfDNA versus cancer-free [*sic*] DNA?

²² See Van Ness Tr. at 111:8-113:13.

²³ See Volik (2016) at 898 (emphasis added).

²⁴ *Id.* at 906(emphasis added).

- A. There may be differences because the multiplexing does involve sample preparation. It does involve understanding the limits of detection. How many cycles of multiplex PCR might be needed? *So, yes, there are differences.*
- Q. Would you say those same differences would apply to performing multiplex PCR to amplify fetal cell-free DNA versus cancer cell-free DNA?
- A. *I think every application has its unique challenges* in terms of sample access, preparation that influence how the multiplex is being done.
- Q. So the answer's yes?
- A. *I think all of those applications, yes,* can have differences in protocols that might be required for successful multiplex amplification.
- Q. And by "applications," you mean cell-free DNA from a fetus versus cell-free DNA from an organ transplant versus cell-free DNA from a tumor?
- A. Recognize that the detection of cell-free DNA from each of those sources also requires access to those sources so that you know what you're looking for. So it's not sufficient simply to isolate cell-free DNA. You have to know the sequences of your target in order to be – to analyze that. And those target sequences are going to be present in different quantities in each of those applications.
- Q. I was just asking what you meant by the word "applications." You say, "I think all of those applications, yes, can have differences in protocols that might be required for successful multiplex amplifications." I'm just asking what you meant by "applications," that word.
- A. Okay.

Q. Are you referring to cell-free DNA of different sources?

A. *I'm referring to cell-free DNA as it is applied to different situations, of fetal DNA, cancer DNA, organ transplant DNA.*²⁵

III. SUMMARY OF OPINIONS

A. Claim Construction

23. It is my opinion that one of ordinary skill in the art would have understood each of the terms of the Asserted Claims of the Natera Patents to have their plain and ordinary meaning. In particular, I construe the terms “tagging isolated cell free DNA” (Claim 1 of the '035 Patent) and “amplifying the tagged products one or more times” (Claim 1 of the '035 Patent) to each have a plain and ordinary meaning, which are summarized *infra*.

24. It is my opinion that one of ordinary skill in the art would have understood that as of the priority date of the '035 Patent, and in light of the Specification, the plain meaning of “tagging isolated cell free DNA” encompasses adding one or more adaptor tags to isolated cell free DNA through primer extension or ligation. Dr. Van Ness agreed with this understanding during his deposition.²⁶

25. It is my opinion that one of ordinary skill in the art would have understood that as of the priority date of the '035 Patent, and in light of its specification, the plain

²⁵ See Van Ness Tr. at 64:4-66:6 (objections, court reporter clarification, and colloquy omitted, emphases added).

²⁶ *Id.* at 228:17-232:25.

meaning of “amplifying the tagged products one or more times” encompasses creating at least one copy of the tagged products. Dr. Van Ness agreed with this understanding during his deposition.²⁷ One of ordinary skill in the art would have readily recognized this occurs following at least one cycle of PCR.²⁸

B. Validity

26. As a preliminary matter, I note that Dr. Van Ness does not contend that any prior art reference discloses every limitation of the Natera Patent claims—*i.e.*, a “novelty” or “anticipation” challenge under Section 102 of the Patent Act.²⁹ Instead, Dr. Van Ness contends that several references, if modified and combined, render the claims obvious.

27. I disagree with Dr. Van Ness’s contention that the Natera Patents are invalid as obvious under Section 103.³⁰ As explained *infra*, Dr. Van Ness fails to account for technical limitations in prior art approaches and the many challenges inherent in manipulating cfDNA faced by skilled artisans.

28. Dr. Van Ness’s obviousness opinions are wholly conclusory. The Van Ness Declaration points to disparate teachings in the art or a skilled artisan’s knowledge that Dr. Van Ness believes remediate these deficiencies.

²⁷ *Id.*

²⁸ *Id.*

²⁹ *Id.* at 97:11-98:13; *see generally* Van Ness Decl.

³⁰ *See* Van Ness Decl. at ¶¶ 109-220 for the ’454 Patent and ¶¶ 221-303 for the ’035 Patent.

29. Dr. Van Ness also fails to explain why one of skill in the art would have been motivated to modify the known prior art approaches in the specific ways needed to achieve the claimed inventions. Just as critically, Dr. Van Ness fails to explain why one of skill in the art would have reasonably expected to succeed with any particular combined approach. In fact, success was unlikely or at the very least unpredictable in light of well-known obstacles, including using cfDNA, which is fragmented and challenging to manipulate even with known methods.³¹ It was particularly difficult to process samples in a manner that would permit analysis of genetic variants on low-abundance, unpredictable cancer-associated cfDNA. Dr. Van Ness fails to even contend, much less show, that a skilled artisan would have reasonably expected success in achieving the claimed methods given the art's unpredictability. His opinions are informed with the benefit of hindsight knowledge of Natera's claimed invention, which is I understand impermissible in an obviousness analysis.

30. I further disagree with Dr. Van Ness's contention that the Natera Patents are invalid as patent-ineligible under Section 101.³² The claims are directed to patent-eligible subject matter (methods of sample preparation), and recite specific concrete laboratory steps which generate DNA sequences not found in nature, and which require human effort to perform. The recited steps, when considered in an ordered combination as well as separately, are inventive and neither routine or conventional.

³¹ See Section II.

³² See Van Ness Decl. at ¶¶ 304-340 for the '454 Patent and ¶¶ 341-347 for the '035 Patent.

31. The Van Ness Declaration arrives at his patent-eligibility opinions by cherry-picking parts of the claims that Dr. Van Ness contends “involve” a natural phenomenon or abstract idea and disregarding all of the rest of the claims that does not support his opinions. Nor does Dr. Van Ness analyze whether the claimed steps are routine and conventional when they are considered in combination as recited in the claims.

32. In addition, I disagree with Dr. Van Ness’s contention that the Natera Patents are invalid as lacking adequate written description under Section 112.³³ The Natera Patents adequately describe the claimed inventions, and the Van Ness Declaration’s contrary opinions are in my opinion incorrect. Contrary to Dr. Van Ness’s opinion, the ’454 Patent explicitly describes the use of whole genome sequencing.³⁴ Dr. Van Ness opines otherwise only by looking outside the patent to other documents not incorporated into or cited in the patent in an attempt to interpret the patent, which I understand is impermissible in an analysis of adequacy of written description.

33. Dr. Van Ness also contends that the Natera Patents lack adequate description of the claimed methods, including approaches to multiplex PCR that do not require selecting primers to avoid primer dimers. I disagree. As explained *infra*, the Natera Patents adequately describe the claimed inventions and teach strategies for multiplex PCR that not only avoid primer dimers but also avoid other problems such as allelic bias.³⁵ In addition,

³³ *Id.* at ¶¶ 404-426 for both the ’454 Patent and ’035 Patent.

³⁴ *See, e.g.*, ’454 Patent at 112:50-66, 116:20-35.

³⁵ *See, e.g.*, ’035 Patent at 48:30-47.

the '035 Patent adequately describes the claimed invention, including the use of universal tail adaptors.³⁶

34. The Van Ness Declaration never asserts that Dr. Van Ness is offering an opinion on a substantial question of validity with respect to either the '454 or '035 Patents. I have considered the Van Ness Declaration, Dr. Van Ness's deposition transcript, and the cited materials. In my opinion, Dr. Van Ness has failed to identify a substantial question of validity for either Natera Patent. In fact, when properly considered, the evidence confirms the Natera Patents are valid.

C. Inventorship

35. I understand that Dr. Van Ness has opined on the issue of inventorship. I am informed by counsel that Dr. Van Ness's opinions are legally improper and inconsistent with the evidence in the record. I disagree with Dr. Van Ness's contention that the '454 Patent is invalid for improper inventorship.

D. Infringement

36. Based on my review of the materials referenced in my Opening Declaration³⁷, and my personal knowledge and experience in this field, it is my opinion that the accused RaDaR assay infringes, literally and under the doctrine of equivalents, the Asserted Claims of the Natera Patents. I respond to Dr. Van Ness's opinions regarding non-infringement in this Reply Declaration.

³⁶ See, e.g., *id.* at 95:16-23.

³⁷ See Metzker Op. Decl. at § VIII.

37. I disagree with the contention in Dr. Van Ness's Declaration that the accused RaDaR assay does not infringe the Asserted Claims of the Natera Patents.³⁸ As I explained in my Opening Declaration, the accused RaDaR assay literally infringes the Asserted Claims of the Natera Patents.³⁹ Dr. Van Ness's noninfringement theories require, as a foundation, claim constructions that deviate from the plain and ordinary meaning of the asserted claim terms. However, Dr. Van Ness has not offered an opinion on the construction of any claim terms except for the term "amplifying."⁴⁰ I have been informed and understand from counsel that Dr. Van Ness has thus waived an interpretation that differs from the plain and ordinary meaning of these claim terms. I also disagree with Dr. Van Ness's construction of "amplifying" because it directly contradicts the plain language of the claim. As explained *infra*, because the Van Ness Declaration's interpretation of these terms is incorrect, his opinions on noninfringement are likewise incorrect.

38. Even the Van Ness Declaration's interpretations of certain claim terms were adopted by the Court, it is my opinion that the accused RaDaR assay still infringes the Asserted Claims of the Natera Patents under the doctrine of equivalents.

E. Signatera

39. I disagree with Dr. Van Ness's contention that Natera's Signatera assay does not practice the Natera Patents. As I explained in my Opening Declaration, Signatera

³⁸ See Van Ness. Decl. at § IX.

³⁹ See Metzker Op. Decl. at § VIII.

⁴⁰ See Van Ness Decl. at § VII; *see also* § III.A *supra*.

practices at least Claim 1 of the '454 Patent and Claim 1 of the '035 Patent.⁴¹ My analysis is supported by Natera's internal technical documentation, which the Van Ness Declaration failed to consider. Moreover, Dr. Van Ness testified that he had not reviewed any of Natera's standard operating procedures.⁴² These materials, when properly considered, confirm that Signatera practices each of the Natera Patents. To the extent that Dr. Van Ness offers any additional opinions regarding Signatera, I reserve the right to supplement my analysis and opinions.

IV. NATERA PATENTS

40. I have previously described the Natera Patents in my Opening Declaration in this case⁴³ and incorporate that description again here in this Reply Declaration. For ease of reference, I include *infra* the claims of the Natera Patents challenged in the Van Ness Declaration.

A. U.S. Patent No. 11,530,454

41. Claim 1 of the '454 Patent recites:

A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:

performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;

⁴¹ See Metzker Op. Decl. at ¶¶ 124-138.

⁴² See Van Ness Tr. at 246:11-20.

⁴³ See Metzker Op. Decl. at ¶¶ 48-53.

performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases,

wherein the target loci are amplified together in the same reaction volume; and

sequencing the amplicons to obtain sequence reads, and

detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads,

wherein the sequencing has a depth of read of at least 50,000 per target locus.

42. Claim 8 of the '454 Patent recites:

The method of claim 1, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

43. Claim 11 of the '454 Patent recites:

The method of claim 1, wherein the method further comprises performing barcoding PCR prior to the sequencing.

B. U.S. Patent No. 11,519,035

44. Claim 1 of the '035 Patent recites:

A method for amplifying and sequencing DNA, comprising:

tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products,

wherein the isolated cell-free DNA is isolated from a blood sample collected from a subject who is not a pregnant women;

amplifying the tagged products one or more times to generate final amplification products,

wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume,

wherein one of the amplifying steps introduces a barcode and one or more sequencing tags; and

sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products,

wherein the plurality of SNP loci comprises 25-2,000 loci associated with cancer.

45. Claim 12 of the '035 Patent recites:

The method of claim 1, wherein the one or more universal tail adaptors comprise a first universal tail adaptor and a second universal tail adaptor.

46. Claim 13 of the '035 Patent recites:

The method of claim 12, wherein tagging the cell free DNA comprises amplifying the cell free DNA with a first primer comprising the first universal tail adaptor and a second primer comprising the second universal tail adaptor.

C. Priority Dates

47. Dr. Van Ness agrees with Natera's contention that the priority date for the '454 Patent is April 21, 2015 and that the priority date for the '035 Patent is May 18, 2011.⁴⁴ In light of this, and in order to respond to Dr. Van Ness's opinions, I apply the same priority dates for the respective Natera Patents in my analysis in this Reply Declaration, though I

⁴⁴ See Van Ness Decl. at ¶¶ 38-39.

reserve the right to offer opinions regarding different priority dates for the Natera Patents at another time.

D. Level of Ordinary Skill in the Art

48. Dr. Van Ness agrees with my prior definition of one of ordinary skill in the art with respect to the Natera Patents.⁴⁵ I apply that definition⁴⁶ again in my analysis in this Reply Declaration, and I reserve the right to offer another definition, for example, in response to any definition that any NeoGenomics expert may offer at another time.

V. CLAIM CONSTRUCTION

49. It is my opinion that one of ordinary skill in the art would have understood each of the terms of the Asserted Claims of the Natera Patents to have their plain and ordinary meaning. In particular, I construe the terms “tagging isolated cell free DNA” (Claim 1 of the ’035 Patent) and “amplifying the tagged products one or more times” (Claim 1 of the ’035 Patent) to each have a plain and ordinary meaning, which I explain in more detail *infra*.

A. “tagging isolated cell-free DNA” (Claims 1, 13 of the ’035 Patent)

(i) *Intrinsic Evidence*

50. It is my opinion that one of ordinary skill in the art would have understood that as of the priority date of the ’035 Patent, the plain and ordinary meaning of “tagging

⁴⁵ *Id.* at §IV.

⁴⁶ *See* Metzker Op. Decl. at § IV. Throughout this Reply Declaration, I also refer to a POSA as one of ordinary skill in the art.

isolated cell free DNA” encompasses adding one or more adaptor tags to isolated cell free DNA through primer extension or ligation. Dr. Van Ness agreed with this interpretation in his deposition.⁴⁷

51. The '035 Patent describes methods for preparing cell-free DNA samples. These methods include adding tags to the cfDNA, which the patent refers to as “tails.”⁴⁸ Specifically, in a section titled “Mini-PCR,” the '035 Patent describes that “[t]he following Mini-PCR method is desirable for samples containing short nucleic acids, digested nucleic acids, or fragmented nucleic acids, such as cfDNA.”⁴⁹ The '035 Patent describes amplifying cfDNA, which can include “polymorphic” sites of interest, using multiplex PCR with forward and reverse primers. For example, the '035 Patent explains that “[i]n an embodiment, the 3' end of the inner forward and reverse primers are designed to hybridize to a region of DNA upstream from the polymorphic site, and separated from the polymorphic site by a small number of bases.”⁵⁰ As part of this process, the '035 Patent explains that “tails with no homology to the target genome may also be added to the 3-prime or 5-prime end of any of the primers.”⁵¹ According to the '035 Patent, “[t]hese tails facilitate subsequent manipulations, procedures, or measurements.”⁵²

⁴⁷ See Van Ness Tr. at 228:17-232:25.

⁴⁸ See, e.g., '035 Patent at 90:38-91:18.

⁴⁹ *Id.* at 88:53-55.

⁵⁰ *Id.* at 89:39-43.

⁵¹ *Id.* at 90:38-40.

⁵² *Id.* at 90:40-41.

52. The '035 Patent describes the process of adding tails to DNA sequences using PCR. In the exemplary description *infra*, for example, the PCR primers contain a “tail” sequence that is not complementary to and therefore would not interact with the target sequence of interest. The addition of tags can be used to manipulate the cfDNA, for example by serving as a site for PCR amplification.

In an embodiment, the method as it pertains to a single target locus may comprise one or more of the following steps: (1) Designing a standard pair of oligomers for PCR amplification of a specific locus. (2) Adding, during synthesis, a sequence of specified bases with no or minimal complementarity to the target locus or genome to the 5' end of the one of the target specific oligomer. ***This sequence, termed the tail, is a known sequence, to be used for subsequent amplification, followed by a sequence of random nucleotides.***⁵³

53. As one of ordinary skill in the art would have understood from the '035 Patent, the description of “tails” includes adaptor tags. In its Definitions, the '035 Patent describes the use of adaptor tags, which is consistent with the '035 Patent's description of “tails.” As the '035 Patent clarifies, adaptor tags can be added to a DNA sequence (for example) either by using PCR or using ligation.

Universal Adapters, or 'ligation adaptors' or 'library tags' are DNA molecules containing a universal priming sequence that can be covalently linked to the 5-prime and 3-prime end of a population of target double stranded DNA molecules. The addition of the adapters provides universal priming sequences to the 5-prime and 3-prime end of the target population from which PCR amplification can take place,

⁵³ *Id.* at 118:14-23 (emphasis added).

amplifying all molecules from the target population, using a single pair of amplification primers.⁵⁴

Universal Priming Sequence refers to a *DNA sequence that may be appended to a population of target DNA molecules, for example by ligation, PCR, or ligation mediated PCR*. Once added to the population of target molecules, primers specific to the universal priming sequences can be used to amplify the target population using a single pair of amplification primers. Universal priming sequences are typically not related to the target sequences.⁵⁵

54. The '035 Patent explicitly describes adding adaptor tags to a DNA sequence of interest. These adaptors are added to the DNA as part of the process of preparing the DNA sample for subsequent sequencing and analysis. As the '035 Patent explains, these adaptors (*i.e.*, tails) can serve as a basis for (universal) amplification of the DNA sequence. In the exemplary disclosure *infra*, the '035 Patent describes adding the adaptors to the DNA using “target specific” primers that include the adaptor sequences, followed by PCR which attaches the adaptor sequence to the DNA through the amplification process.

In an embodiment, *the targeted sequencing may involve* one, a plurality, or all of the following steps. a) *Generate and amplify a library with adaptor sequences on both ends of DNA fragments*. b) Divide into multiple reactions after library amplification. c) *Generate and optionally amplify a library with adaptor sequences on both ends of DNA fragments*. d) *Perform 1000- to 10,000-plex amplification of selected targets using* one target specific “Forward” primer per target and *one tag specific primer*. e) *Perform a second amplification from this product using* “Reverse” target specific primers and *one (or more) primer specific to a universal tag that was introduced as part of the target specific*

⁵⁴ *Id.* at 42:22-30 (emphases added).

⁵⁵ *Id.* at 42:14-21 (emphases added).

forward primers in the first round. f) Perform a 1000-plex preamplification of selected target for a limited number of cycles. g) Divide the product into multiple aliquots and amplify subpools of targets in individual reactions (for example, 50 to 500-plex, though this can be used all the way down to singleplex. h) Pool products of parallel subpools reactions. i) During these amplifications primers may carry sequencing compatible tags (partial or full length) such that the products can be sequenced.⁵⁶

55. The '120 Patent Pub. (Zimmermann),⁵⁷ which the '035 Patent incorporates by reference, contains additional description of this process, including on cfDNA-containing plasma samples.

The workflow may entail (1) extracting DNA such as plasma DNA, (2) preparing fragment library with universal adaptors on both ends of fragments, (3) amplifying the library using universal primers specific to the adaptors,⁵⁸ ... ('120 Pub. [0213])⁵⁹

56. Relatedly, the '035 Patent explains the usefulness of adding adaptor tags to cfDNA. For example, the '035 Patent describes that “primers are designed to anneal a site a greater distance from the polymorphism than is expected to be present among cell free fetal DNA fragments,” and that these “primers are tagged with a molecule or moiety that can allow selective recognition of the tagged pieces of DNA.”⁶⁰

⁵⁶ *Id.* at 85:47-67 (emphases added).

⁵⁷ See Zimmermann *et al.*, *Highly multiplex PCR methods and compositions*, U.S. Patent Application Publication No. US 2013/0123120 (2013) (the “'120 Patent Pub. (Zimmermann)”).

⁵⁸ See '035 Patent at 86:61-64.

⁵⁹ *Id.* at 250:9-11.

⁶⁰ *Id.* at 91:28-35.

57. Adding adaptor tags to cfDNA would facilitate sequencing the DNA, as “using forward and reverse tails corresponding to forward and reverse sequences required by any of the current sequencing platforms can enable direct sequencing following amplification.”⁶¹ The adaptor tags facilitate high-throughput sequencing in particular. As the ’035 Patent explains, “a 10,000-plex PCR assay pool is created such that forward and reverse primers have tails corresponding to the required forward and reverse sequences required by a high throughput sequencing instrument such as the HISEQ, GAIIX, or MYSEQ available from ILLUMINA.”⁶²

58. Additionally, consistent with the Definitions of adaptor sequences *supra*, the ’035 Patent teaches that “the tails can be used as common priming sites among all amplified targets that can be used to add other useful sequences,” such as a molecular barcode. To achieve this end, “the primer may contain a universal priming sequence designed to allow PCR amplification.”⁶³

59. I have reviewed the prosecution history for the ’035 Patent. From my review, the prosecution history is consistent with my opinion *supra* and I see nothing to indicate that the term “tagging isolated cell free DNA” should be construed to have a different meaning other than its plain and ordinary meaning that I describe *supra*. I see, for example,

⁶¹ *Id.* at 90:48-51.

⁶² *Id.* at 90:60-64.

⁶³ *Id.* at 90:51-59.

that this limitation was not amended and was present in the originally-filed claims (dated July 21, 2020), excerpted *infra*.⁶⁴

1. A method for amplifying and sequencing DNA, comprising:
 - tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products, wherein the isolated cell-free DNA is isolated from a blood sample collected from a subject;
 - amplifying the tagged products one or more times to generate final amplification products, wherein one of the amplifying steps introduces a barcode and one or more sequencing tags; and
 - sequencing a plurality of loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products, wherein the plurality of loci comprises 25-2,000 loci.

(ii) *Extrinsic Evidence*

60. As further support for my opinions, one of ordinary skill in the art's understanding of adaptor tags and their addition to DNA sequences is shown in evidence extrinsic to the '035 Patent itself.

61. One of ordinary skill in the art would have readily recognized that for sequencing on most platforms, including some of the most widely-used models (*e.g.*, Illumina sequencers), sequencing adaptors are always required.⁶⁵ Briefly, adding these adaptors is necessary for sequencing DNA, which can include amplicons of cfDNA. Sequencers contain complementary sequences that recognize and bind the adaptor

⁶⁴ See NAT-NEO-00001599-NAT-NEO-00003251 (Prosecution History of the '035 Patent) at NAT-NEO-00001615.

⁶⁵ See Ex. 11, Meyer & Kircher, *Illumina sequencing library preparation for highly multiplexed target capture and sequencing*, COLD SPRING HARBOR PROTOCOLS, pdb.prot5448 pp. 1-10 (2010) ("Meyer (2010)").

sequences and hold them in place for the sequencing reactions to occur and be detected in the machine. When adaptor-tagged pieces of DNA (including amplicons of cfDNA) are introduced in the sequencer, the adaptors bind to complementary sequences in the instrument's *flow cell*. The pieces of DNA are held in place for sequencing and further analysis.

B. “amplifying the tagged products one or more times” (Claim 1 of the ’035 Patent)

(i) *Intrinsic Evidence*

62. It is my opinion that one of ordinary skill in the art would have understood that as of the priority date of the ’035 Patent, the plain meaning of “amplifying the tagged products one or more times” encompasses creating at least one copy of a tagged product, which one of ordinary skill in the art would have readily recognized occurs in multi-cycle PCR following the addition of a tag after the first cycle of PCR. Dr. Van Ness agreed with this in his deposition.⁶⁶

63. The ’035 Patent specification describes how DNA sequences of interest are tagged after a PCR cycle, and then those tagged products are amplified in one or more further PCR cycles. For context, consistent with one of ordinary skill in the art’s general understanding of the PCR reaction, the ’035 Patent describes that “[i]n some embodiments, most or all of the primers,” which are single-stranded DNA molecules that bind complementary sequences on the DNA sequence of interest, “are extended to form

⁶⁶ See Van Ness Tr. at 220:1-16.

amplified products” via the PCR method.⁶⁷ In consequence, the primers—including any tag sequences within the primer—are “consumed” via incorporation into the DNA strand being replicated during a PCR cycle. The ’035 Patent describes this process as “conversion” of the primer into the amplicon (which also contains the DNA sequence). “Having all the primers consumed in the PCR reaction increases the uniformity of amplification of the different target loci since the same or similar number of primer molecules are converted to target amplicons for each target loci.”⁶⁸

64. The ’035 Patent describes tagging DNA in a single PCR reaction in the context of explaining how to enrich the fraction of cell free DNA from a fetus isolated from a sample of plasma obtained from a pregnant woman. I note in this situation, the fetal cfDNA is typically present at relatively low levels and challenging to detect. One of ordinary skill in the art would have understood from the ’035 Patent’s written description that its methods regarding fetal cfDNA are applicable to other plasma samples containing cfDNA samples where the DNA sequences of interest are low-abundance (*e.g.*, as a “contaminant”). This could be in the case of a cancer subject’s plasma DNA that often contains tumor-associated cfDNA at low levels.⁶⁹

⁶⁷ See ’035 Patent at 83:8-9.

⁶⁸ *Id.* at 83:9-13.

⁶⁹ The ’035 Patent explain, for example, that “[t]he methods described herein may be used for a number of purposes where there is a target set of DNA that is mixed with an amount of contaminating DNA. In some embodiments, the target DNA and the contaminating DNA may be from individuals who are genetically related. For example, genetic abnormalities in a fetus (target) may be detected from maternal plasma which contains fetal

65. In the description of this enrichment process, the '035 Patent describes tagging DNA using tagged primers in a single cycle of PCR. Specifically, the '035 Patent explains that the primers used for this enrichment process “are designed to anneal [bind] a site a greater distance from the polymorphism than is expected to be present among cell free fetal DNA fragments.”⁷⁰ The '035 Patent explains that “[t]hese distal primers are tagged with a molecule or moiety that can allow selective recognition of the tagged pieces of DNA.”⁷¹ '035 Patent describes that “[t]hese primers may be used in a *one cycle* multiplex PCR reaction *prior to multiplex PCR* of the target polymorphic loci.”⁷² Accordingly, the '035 Patent teaches that, in the very first PCR cycle, tags on primers are appended to the DNA sequence being amplified. The tagged products are thereafter amplified one or more times in subsequent PCR cycles.

66. Although the process described *supra* relates to tagging DNA for removal from a sample, one of ordinary skill in the art would have understood that it applies equally

(target) DNA and also maternal (contaminating) DNA; the abnormalities include whole chromosome abnormalities (e.g. aneuploidy) partial chromosome abnormalities (e.g. deletions, duplications, inversions, and translocations), polynucleotide polymorphisms (e.g. STRs), single nucleotide polymorphisms, and/or other genetic abnormalities or differences. In some embodiments, the target and contaminating DNA may be from the same individual, but where the target and contaminating DNA are different by one or more mutations, for example in the case of cancer.” *Id.* at 92:16-32.

⁷⁰ *Id.* at 91:28-31.

⁷¹ *Id.* at 91:33-35.

⁷² *Id.* at 91:31-33 (emphasis added).

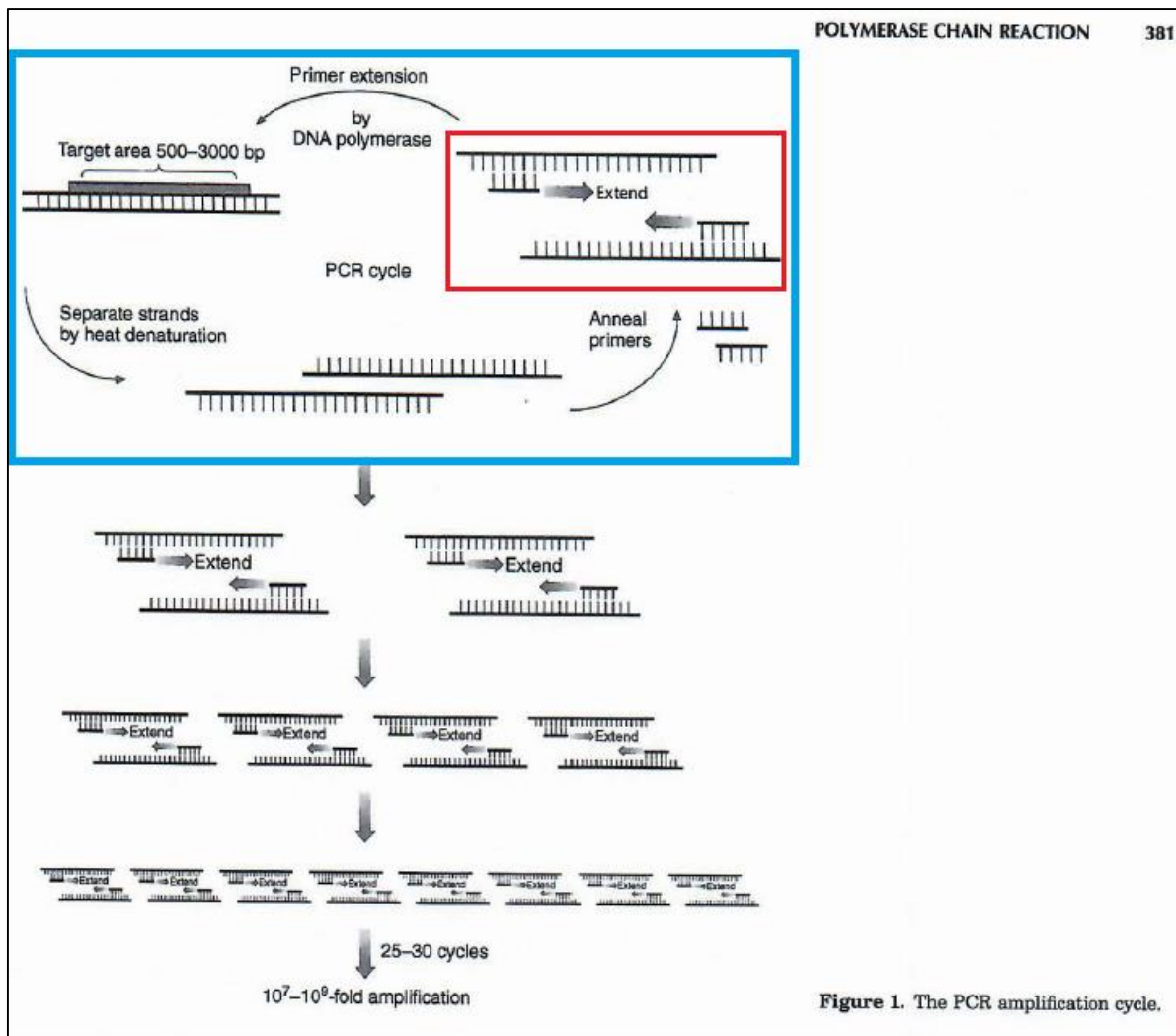
to tagging DNA that will be further manipulated. In either case, the addition of a tag to DNA via PCR occurs at the very first cycle that PCR is performed.

67. I have reviewed the prosecution history for the '035 Patent. Although I do not see anything specifically in the prosecution that addresses the term “amplifying the tagged products one or more times,” from my review, the prosecution history is consistent with my opinion. I see nothing to indicate that this term should be construed to have a different meaning other than its plain and ordinary meaning as described *supra*.

(ii) *Extrinsic Evidence*

68. One of ordinary skill in the art’s understanding of PCR and the addition of a tag to a DNA sequence of interest after a single cycle is also shown in evidence extrinsic to the '035 Patent itself. For example, I previously explained the fundamentals of PCR in a book chapter I authored, which was published in 2006 and discussed during my deposition.⁷³ The figure *infra* depicts how within a single cycle of PCR (blue outline), both a forward and reverse primer can hybridize to different ends of a template DNA molecule after it is denatured into two single strands. A DNA polymerase can then build two complementary strands (red outline). I explain this figure in greater detail *infra*.

⁷³ See Ex. 6, Metzker & Caskey, *Polymerase Chain Reaction*, In *ENCYCLOPEDIA OF MEDICAL DEVICES AND INSTRUMENTATION*, Second Edition, Volume 5, pp. 380-387 (2006) (“Metzker (2006)”) (cited as Ex. 7 in the September 26, 2023 Deposition of Michael Metzker Transcript (Dkt. 116-6)).



69. During the first cycle of PCR (blue outline), a template strand of DNA is denatured into two single strands by heat or chemical denaturant. At a different step in the same cycle, a forward primer will hybridize to the 5' end of one single stranded template DNA and DNA polymerase will build a complementary strand, extending from 5' to 3' (directionality is with reference to the template DNA strand). Also in the same cycle, a reverse primer will hybridize to the 3' end of the other single stranded template DNA and

DNA polymerase will build a complementary strand, extending from 5' to 3' (directionality is with reference to the newly synthesized complementary DNA strand).

70. Therefore, a single cycle of PCR can introduce up to *two tags* (one on each strand of the amplified DNA product). The subsequent cycles of PCR amplify the target region of interest, thereby performing targeted amplification of already tagged DNA.

(iii) *Dr. Van Ness' construction of "amplifying" contradicts the plain language of the claims*

71. The Declaration of Van Ness opines that the term "amplifying" in Claims 1 and 13 of the '035 Patent should be construed as "increasing the number copies of a molecule, such as a molecule of DNA."⁷⁴ I agree that this interpretation is consistent with the plain and ordinary meaning of "amplifying." I disagree with Dr. Van Ness's use of "copies," however, being "not just the copies that result from the initial copying of the original sample DNA strand, but also the copies of those copies and any subsequent copies."⁷⁵ In his interpretation, Dr. Van Ness treats "amplifying ... one or more times" as recited in the claim to mean a completed multi-cycle PCR process, overlooking the well-accepted understanding of one of ordinary skill in the art that PCR is a process comprised of iterative amplification cycles. Dr. Van Ness re-defines "amplifying ... one or more times" to mean completing all steps of a multi-cycle PCR. According to Dr. Van Ness,

⁷⁴ See Van Ness Decl. at ¶ 65.

⁷⁵ *Id.*

“copies”—which is a term not recited at all in the claim—do not result until the end of all amplification cycles of the *entire* PCR process. I disagree.

72. At the end of one amplification cycle in the PCR process, one of ordinary skill would have understood that the output (amplified products) is used as the input for the next amplification cycle, thus creating copies of these amplified products. Contrary to the opinion offered in the Van Ness Declaration, Dr. Van Ness in fact agreed with this basic understanding of how PCR works in his deposition.⁷⁶ This process is iterative throughout the PCR process such that copies are being generated at the end of each amplification cycle. Dr. Van Ness also agreed with this understanding of PCR in his deposition.⁷⁷

73. Dr. Van Ness adopts an overly narrow opinion and ignores the plain language of the claim itself, which as described *supra*, provides context for “amplifying” and ***does not*** recite the word “copies” that is the entire basis for Dr. Van Ness’s opinion. Claim 1 of the ’035 Patent recites “amplifying the tagged products *one or more times*.” One of ordinary skill in the art would have understood that once cell-free DNA has been tagged (as required earlier in the claim), the tagged cell-free DNA serves as a template for amplification and is amplified one or more times. Dr. Van Ness’s deposition testimony does not refute this understanding.⁷⁸ Even using Dr. Van Ness’s “copies” terminology,

⁷⁶ Van Ness Tr. at 220:1-16.

⁷⁷ *Id.* at 219:13-15.

⁷⁸ *Id.* at 228:17-232:25.

amplifying the tagged cell-free DNA one time—in one amplification cycle of the PCR—will generate a copy of the tagged cell-free DNA, satisfying the claim limitation. For this reason, I disagree with Dr. Van Ness’s overall construction of “amplifying” because it directly contradicts the plain language of the claim.

VI. THE NATERA PATENTS ARE NOT OBVIOUS UNDER SECTION 103

74. I have reviewed Dr. Van Ness’s opinions regarding purported obviousness of the Natera Patents under Section 103.⁷⁹ I disagree with his opinions including for all the reasons explained *infra*. In providing my opinions in reply, I focus my analysis on certain aspects of Dr. Van Ness’s contentions that in my opinion are incorrect or deficient and fail to support his conclusions. I reserve the right to offer additional opinions with respect to the Van Ness Declaration at another time. To be clear, the absence of any opinion expressed in this Reply Declaration should not be assumed to be an agreement with those opinions asserted in the Van Ness Declaration or a representation that I will not offer opinions in the larger case after this preliminary injunction proceeding.

75. **No motivation to combine or modify the cited references with a reasonable expectation of success in arriving at the claimed inventions.** As a preliminary matter, I have been informed and understand that an obviousness analysis requires an evaluation of whether one of ordinary skill in the art would have been motivated to combine or modify the cited references with a reasonable expectation of success in

⁷⁹ See Van Ness Decl. at ¶¶ 109-220 for the ’454 Patent and ¶¶ 221-303 for the ’035 Patent.

achieving the claimed invention, based on the teachings of the art in the cited references and the knowledge of a skilled artisan. This analysis of a motivation to combine or modify with a reasonable likelihood of success does not appear anywhere in the opinions asserted in the Van Ness Declaration—Dr. Van Ness does not address this legal requirement of obviousness at all. In consequence, Dr. Van Ness’s opinions are inadequately supported, at a minimum, because they fail to address all of the legal elements for establishing obviousness. Because a motivation to combine/modify with a reasonable expectation of success is nevertheless part of the obviousness analysis, I offer my opinions regarding why one of ordinary skill would not have been motivated to combine or modify the cited references and would not have reasonably expected success in achieving the Natera Patents’ inventive methods.

76. **No consideration of objective indicia of non-obviousness.** I also have been informed and understand that an obviousness analysis requires consideration of objective indicia of the claims’ non-obviousness when those indicia are present. Indeed, I understand that these objective indicia may be some of the most probative evidence of a patent’s claims’ non-obviousness. These objective indicia can include, for example, commercial success, unexpected results, failure of others, long felt but unmet need, and industry praise. Dr. Van Ness fails to analyze any objective indicia regarding the Natera Patents’ claims.

77. **Dr. Van Ness impermissibly relies on hindsight.** I have been informed and understand from counsel that the use of hindsight reconstruction of the claimed invention, by picking and choosing among isolated disclosures in the prior art, is impermissible in an

obviousness analysis. I have been further informed and understand that showing a teaching or motivation to combine prior art references is a defense to guard against the impermissible use of hindsight. I am also informed and understand that the consideration of objective indicia of non-obviousness is another way to avoid hindsight bias.

78. As explained further *infra*, Dr. Van Ness's opinions reflect the use of hindsight bias in reconstructing the Natera Patents' claimed inventions rather than the objective understanding of a skilled artisan guided only by the prior art and the then-accepted wisdom in the field. Specifically, Dr. Van Ness selects from among disparate disclosures in the prior art and the knowledge of one of ordinary skill in the art to support his opinions that the Natera Patents' claimed methods would have been obvious. However, the Van Ness Declaration does not articulate how, as of the patents' priority date(s), one of ordinary skill in the art would have been motivated to combine the teachings or understandings selected by Dr. Van Ness and reasonably expected to succeed in doing so in light of known challenges in the art. Moreover, as noted *supra*, the Van Ness Declaration's invalidity positions do not address objective indicia of non-obviousness.

79. I do not address the validity of the challenged dependent claims of the Natera Patents *infra* because they are not obvious at least for the reasons that the independent claims are not obvious. Dr. Van Ness has failed to establish that the independent claims would have been obvious for the reasons explained *infra*, and his opinions concerning the dependent claims are likewise flawed. I reserve the right to offer additional opinions concerning the dependent claims' validity at another time.

A. The '454 Patent Is Not Obvious

80. As Dr. Van Ness admitted in deposition, he is not opining that the '454 Patent is invalid under Section 102 of the Patent Act, which I understand and am informed by counsel precludes patentability if a prior art reference discloses each and every claim limitation, arranged as in the claim, either expressly or inherently.

Q. And you're – you don't offer any opinions that the '454 patent claims are invalid under Section 102 for anticipation, correct? All your prior art opinions are for obviousness under 103?

A. *That is correct. I'm offering invalidity based on obviousness.*⁸⁰

(i) *Forshew (2012) Does Not Render the '454 Patent Claims Obvious*

81. I disagree with Dr. Van Ness's opinions that the Asserted Claims of the '454 Patent are rendered obvious by Forshew (2012) in view of the skill and knowledge of a skilled artisan.⁸¹ At least for the reasons explained *infra*, Dr. Van Ness has failed to demonstrate that the '454 Patent claims are obvious over Forshew (2012), a reference already considered by the Patent Examiner during prosecution.

82. For the claims to be obvious, *inter alia*, it must have been obvious to one of ordinary skill in the art to have performed (ultra-deep) sequencing to “a depth of read of at least 50,000 per target locus,” as recited in Claim 1[c] of the '454 Patent,⁸² in the course of

⁸⁰ See Van Ness Tr. at 94:24-95:4 (emphasis added).

⁸¹ See Van Ness Decl. at § XA.

⁸² See '454 Patent at 171:39-43.

performing the other method steps. Dr. Van Ness's opinions regarding the value and utility of performing this sequencing are deficient at least because, as explained *infra*, one of ordinary skill in the art would not have understood the claimed ultra-deep $\geq 50,000$ sequencing depth to be something that would remediate the admitted limited sensitivity described in Forshew (2012)⁸³ and elsewhere.⁸⁴

83. One of ordinary skill in the art therefore would not have expected ultra deep sequencing to a depth of read of at least 50,000 per target locus to enable the Forshew (2012) method to analyze rare mutations in cfDNA. My analysis here focuses on Claim 1[c] of the '454 Patent. However, I reserve the right to offer additional opinions regarding the '454 Patent in view of Forshew (2012) alone or in combination with other references.

(a) **Claim 1[c] of the '454 Patent**

sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the sequencing has a depth of read of at least 50,000 per target locus.

A) Forshew (2012) Does Not Teach A Read Depth Greater Than 7,700 for Analyzing cfDNA

⁸³ See Forshew (2012) at 10. “A *current limitation of TAm-Seq is the detection limit* compared to assays that target individual loci (2, 3, 7, 40), which have been shown to detect two to three orders of magnitude lower frequencies.” (emphasis added).

⁸⁴ See Volik (2016) at 903. “[*The Tam-seq approach*] afforded the opportunity to quantify mutant DNA at many loci in each patient but *had a relatively low sensitivity for mutations below approximately 1%–2%.*” (emphases added).

84. As a preliminary matter, I note that the Van Ness Declaration does not contend that Forshew (2012) teaches each and every limitation of the '454 Patent claims. In particular, the Van Ness Declaration does not dispute that Forshew (2012) does not explicitly teach achieving the ultra-deep sequencing to “a depth of read of at least 50,000 per target locus.”⁸⁵ In deposition, Dr. Van Ness confirmed that “I comment that Forshew had a depth of 15,400. So he did not directly meet that claim in his publication of 50,000.”⁸⁶

85. I agree that Forshew (2012) does not teach the claimed read depth of “at least 50,000 per target locus.” I disagree, however, that Forshew (2012) teaches a read depth of 15,400 in amplifying cfDNA. In fact, Forshew (2012) teaches amplifying cfDNA to an order of magnitude less—*no more than* a mean read depth of 7,700.⁸⁷

86. The Van Ness Declaration asserts that Forshew (2012) teaches sequencing cfDNA to a depth of 15,400 based on the statement that Forshew (2012) “amplified DNA from each sample in duplicate.”⁸⁸ Here, however, the Van Ness Declaration misinterprets disparate disclosures in Forshew (2012). In fact, the “duplicate” amplification suggested by Dr. Van Ness does not involve cfDNA. Rather, that passage from Forshew (2012)

⁸⁵ See Van Ness Decl. at ¶¶ 141-143.

⁸⁶ See Van Ness Tr. at 97:11-99:21, specifically citing 98:5-9 (emphasis added). However, Dr. Van Ness would not agree with the proposition that Forshew (2012) “doesn’t itself disclose all the claim elements” of the '454 Patent. Van Ness Tr. 95:17-23. To the extent Dr. Van Ness contends that Forshew (2012) discloses all the claim elements of the '454 Patent, I disagree as explained herein.

⁸⁷ See Forshew (2012) at Table 1, column titled “Mean depth (sequencing reads).”

⁸⁸ See Van Ness Decl. at ¶¶ 141-143; *see also* Forshew (2012) at 3.

involves genomic DNA (“gDNA”) isolated from formalin-fixed, paraffin-embedded (FFPE) tumor specimens, which one of ordinary skill in the art would have understood is *not* cfDNA. Unlike cfDNA, which is present in plasma, gDNA is isolated from intact solid tissues and has not undergone fragmentation and degradation.

87. Specifically, Forshew (2012) teaches the use of “TAm-Seq to sequence DNA extracted from 47 formalin-fixed, paraffin-embedded (FFPE) tumor specimens of ovarian cancers.”⁸⁹ Forshew (2012) further explains that “[w]e amplified DNA from each [ovarian tumor] sample in duplicate, tagging each replicate with a different barcode.”⁹⁰ This passage of Forshew (2012) does not teach duplicate amplification or sequencing those duplicated amplified samples using cfDNA isolated from plasma.

88. In fact, as reflected in Table 1 (reproduced *infra*), Forshew (2012) only teaches sequencing cfDNA *from plasma samples* to a maximum read depth of 7,700, as highlighted in the red box *infra*, with most of the applied read depths falling far lower (*e.g.*, 4,200).⁹¹

⁸⁹ See Forshew (2012) at 3, citing to Table S3, not Table 1 as suggested in the Van Ness Decl.

⁹⁰ *Id.*

⁹¹ *Id.* at Table 1 (red box added).

Patient ID	Age at diagnosis	Time elapsed since surgery (months); number of previous lines of chemotherapy	CA125 (U/ml)	Plasma per amplification reaction (µl)	Gene	Mutation and base change (genome build hg19)	Protein change	Detected in FFPE	Mean depth (sequencing reads)	Mean AF using TAM-Seq	Mean AF using digital PCR	
8	60	13; 1	2122	50	TP53	17:7577120	C>T	p.R273H	Y	5000	0.09	0.10
12	62	27; 3	365	50	TP53	17:7577579	G>T	p.Y234*	Y	5000	0.10	0.08
14	58	50; 3	260	120	TP53	17:7578212	G>A	p.R213*	Y	5800	0.15	0.12
25	61	9; 1	944	110	TP53	17:7578404	A>T	p.C176S	Y	4800	0.04	0.08
27 [†]	68	15; 1	1051	90	TP53	17:7578262	C>G	p.R196P	Y	7700	0.06	0.14
					EGFR	7:55259437	G>A	p.R832H	N	5700	0.06	0.05
31	64	12; 1	313	30	TP53	17:7578406	C>T	p.R175H	Y	4500	0.44	0.56
46	56	30; 2	1509	30	TP53	17:7578406	C>T	p.R175H	Y	4200	0.23	0.30

Specifically, Table 1's legend in Forshew (2012) discloses "[m]utations identified by TAM-Seq in plasma samples from seven ovarian cancer patients," for which the highest mean depth of read is 7,700 (Patient ID 27). Forshew (2012) does not provide any teachings or suggestion to increase the sequencing read depth for cfDNA samples to the claimed levels of 50,000 and, as explained *infra*, a skilled artisan would not have been motivated to duplicate or otherwise increase the read depth to detect rare genetic variants in plasma ctDNA as there would have been no reasonable expectation of success given the known technical limitations of Forshew (2012)'s approach. If anything, Forshew (2012) would encourage a skilled artisan to decrease read depth because the majority of read depths Forshew (2012) applied were far below 7,700, going down to the low 4,000's.

B) It Would Not Have Been Obvious to Combine the Claimed Read Depth with Forshew's (2012)'s Method

89. The Van Ness Declaration asserts that "one would have understood Forshew (2012) to teach sequencing depths in excess of 15,400, including 50,000 and beyond."⁹²

⁹² See Van Ness Decl. at ¶ 142.

Relatedly, the Van Ness Declaration asserts that a “POSA would have understood that increasing sequencing depth increases sensitivity in detecting SNPs and it would be routine to increase sequence read depth to obtain greater sensitivity as required.”⁹³ I disagree with each of these contentions.

90. One of ordinary skill in the art would not have been motivated to combine the claimed depth of read with the other claimed method steps and have reasonably expected success sequencing to “a depth of read of at least 50,000 per target locus” (nearly 10x higher than Forshew (2012)’s mean read depth) to detect rare genetic variants such as SNVs, in light of the technical limitations of Forshew (2012)’s method. Specifically, sequencing to the claimed read depth—sometimes called “ultra-deep sequencing”—would not have remedied the limited sensitivity of Forshew (2012)’s approach to detect rare genetic variants, as explained further *infra*.

91. As an initial matter, Forshew (2012) admits the limited sensitivity of its method to detect rare genetic variants.

A current limitation of TAm-Seq is the detection limit compared to assays that target individual loci (2, 3, 7, 40), which have been shown to detect two to three orders of magnitude lower frequencies. Our approach may be sufficient for analyzing plasma from patients with certain advanced cancers, but further improvement may be necessary before this method can be more widely used in the clinic.⁹⁴

⁹³ *Id.* at ¶ 144.

⁹⁴ *See* Forshew (2012) at 10 (emphasis added).

In the context of plasma samples from cancer patients, at best, Forshew (2012) posits merely that its methods “may be sufficient” for use with patient samples containing abundant circulating tumor cfDNA (*e.g.*, patients with advanced cancers). But one of ordinary skill in the art would have understood from Forshew (2012) that its method would not be sensitive enough to detect genetic variants in low-abundance ctDNA in many other kinds of cancers (*e.g.*, in plasma from patients with early-stage cancers, or “minimal residual disease” in the case of patients whose cancers have been surgically excised or treated). Thus, the limitations of Forshew (2012)’s approach would have impaired the ability of a skilled artisan to identify rare tumor-associated SNPs in cfDNA isolated from a plasma sample, as Forshew (2012) itself recognizes.

92. In order for the ’454 Patent claims to be obvious, *inter alia*, it must have been obvious to one of ordinary skill in the art that the claimed ultra-deep sequencing—*i.e.*, the specific $\geq 50,000$ level recited—would have improved Forshew (2012)’s approach and permit detection of rare mutations. But the Forshew (2012) approach alone would not be effective on either score, as one of ordinary skill in the art would have recognized, and therefore the skilled artisan would not have reasonably expected success with the Forshew (2012) strategy.

93. Specifically, the limitations of Forshew (2012)’s approach arise because the errors associated with Forshew (2012)’s sample library preparation and sequencing workflow exceed the technique’s ability to detect rare genetic DNA variants, as explained

in greater detail *infra*. Under the Forshew (2012) approach, the rare variants are lost in the “noise” associated with the abundant errors found in the sequence read data.

1) Ultra-Deep Sequencing Does Not Enable Detection of Rare Genetic Variants that Fall Below the Sequencing Workflow Error Rate

94. Here, I provide background and context for my opinions regarding Forshew (2012). Prior to 2015, there were many challenges in using next-generation sequencing (“NGS”), sometimes also referred to as “massively parallel sequencing,” to process samples and facilitate the analysis of tumor-associated genetic variations including single nucleotide variants (“SNVs”), copy number variations (“CNVs”), and chromosomal rearrangements. Even relatively high-fidelity sequencing platforms were known to introduce sequencing errors at a rate of $\geq 0.1\%$.⁹⁵

95. Additional erroneous nucleotide changes could also be introduced during the PCR amplification steps of sequencing library preparations. For example, Schmitt (2012) explains that base-specific mutagenic DNA damage followed by polymerase misincorporation against such damaged base contributes the error rate of the sequencing process.

Base-specific mutagenic DNA damage is a likely explanation of these imbalances. Excess G→T mutations are consistent with the oxidative product 8-oxo-guanine (8-oxo-G) causing first round PCR errors and artifactual G→T mutations. DNA polymerases, including those commonly used in PCR, have a strong tendency to ***insert adenine opposite 8-oxo-G*** (35, 36),

⁹⁵ See Ex. 3, Glenn, *Field guide to next-generation DNA sequencers*, MOLECULAR ECOLOGY RESOURCES 11:759–769 (2011) (“Glenn (2011)”) at Table 3.

and misinsertion of A opposite 8-oxo-G would result in ***erroneous scoring of a G→T mutation***. Likewise, the excess C→T mutations are consistent with ***spontaneous deamination of cytosine to uracil*** (37), a particularly common DNA damage event that results in ***insertion during PCR of adenine opposite uracil and erroneous scoring of a C→T mutation***.⁹⁶

Such accumulation of potential false positive errors by both PCR and sequencing was known to limit the reliable analysis of true variants that occur with <1% frequency.

96. These technical limitations were recognized in the art. For example, Kinde (2011) explains that massively parallel sequencing (sometimes also referred to as “next generation sequencing” or “NGS”) could not have been used generally to analyze rare variants because of high error rates associated with the sequencing workflow:

[M]assively parallel sequencing cannot generally be used to detect rare variants because of the high error rate associated with the sequencing process. For example, with the commonly used Illumina sequencing instruments, this error rate varies from ~1% (31, 32) to ~0.05% (33, 34), depending on factors such as the read length (35), use of improved base-calling algorithms (36–38), and the type of variants detected (39). Some of these errors presumably result from mutations introduced during template preparation, during the preamplification steps required for library preparation, and during further solid-phase amplification on the instrument itself. Other errors are due to base misincorporation during sequencing and base calling errors. Advances in base calling can enhance confidence (e.g., refs. 36–39), but instrument-

⁹⁶ See Ex. 4, Schmitt *et al.*, *Detection of ultra-rare mutations by next-generation sequencing*, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES 109: 14508-14513 (2012) (“Schmitt (2012)”) at 14510 (emphases added).

based errors are still limiting, particularly in clinical samples wherein the mutation prevalence can be $\leq 0.01\%$ (11).⁹⁷

97. Additionally, prior to the Natera Patents, skilled artisans understood that although increasing read depth⁹⁸ could potentially increase sensitivity, it would not increase the sensitivity beyond the limit of detection based on the error rate. For that reason, “ultra-deep” sequencing of 30,000 reads or more per locus was uncommon and unnecessary for most sequencing efforts. Ultra-deep sequencing was also generally undesirable as it was recognized as expensive and not cost-effective for routine use.

98. Accordingly, one of ordinary skill in the art would have recognized that the sensitivity of preparing samples of and analyzing variants associated with a patient’s cancer, particularly when using cfDNA as source material, would have been hampered by errors introduced during the sample preparation process as well as by the fragmented and variable nature of the cfDNA itself. Such errors could be introduced at both the amplification and sequencing steps of preparing cfDNA preparations.

99. Even after 2014-2015, the field continued to recognize that methods of sample processing and analyzing cancer-associated variant loci were hampered by the lack

⁹⁷ See Ex. 5, Kinde et al., *Detection and quantification of rare mutations with massively parallel sequencing*, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES 108:9530-9535 (2011) (“Kinde”) at 9530 (emphasis added).

⁹⁸ The ’454 Patent explains, for example, that “the depth of read is the number of sequencing reads that map to a given locus” and, “for the depth of read of a locus, the depth of read is the number of reads measured by the sequencer mapping to that locus. In general, the greater the depth of read of a locus, the closer the ratio of alleles at the locus tend to be to the ratio of alleles in the original sample of DNA.” ’454 Patent at 99:66-100:9.

of sensitivity of the available techniques to analyze variants in cfDNA. By way of example, Volik (2016) provides:

Broadly, current approaches for detection of tumor aberrations in cfDNA can be divided into two categories: methods targeting specific changes and methods allowing detection of all possible aberrations in DNA (including targeted and whole exome/genome sequencing). The latter next-generation sequencing (NGS)-based options offer numerous potential benefits for observing clonal differences in tumor cell populations, an advantage that until very recently *was offset by more limited sensitivity and specificity*.⁹⁹

While these early observations highlighted many possibilities for using ctDNA as a noninvasive approach to analyze tumor genomes, *sufficiently sensitive and specific laboratory techniques to fully leverage this potential were not yet developed*.¹⁰⁰

As we have demonstrated earlier, the mutant VAF in patients' cfDNA can go as low as 0.01%. Clearly, any strategy to suppress sources of errors thereby increasing accuracy in detecting mutant DNA molecules is important for cfDNA analysis. *This problem is equivalent to an extreme case of identifying low-abundance mutations in tissue samples, which has also proven difficult for NGS methods*.¹⁰¹

While targeted assays can be fruitful in the clinical setting, sequence-based approaches offer clear advantages in terms of flexibility of coverage and the ability to detect a wide range of aberrations in tumor genomes. This flexibility will be especially important for managing metastasis and resistance to therapy; widely recognized to be among the most important problems in cancer management. Resistance to therapy can be driven by a wide range of genomic aberrations such as point mutations and copy number aberrations. Moreover, resistant

⁹⁹ See Volik (2016) at 901 (emphasis added).

¹⁰⁰ *Id.* at 898 (emphasis added).

¹⁰¹ *Id.* at 905 (emphasis added).

subclones can constitute a very small proportion of the tumors total clonal population until the selective pressure of therapy leads to their rapid expansion. Clearly, the early detection of resistant clones requires sensitivity to detect such events. However, *this requires minimizing noise in the ctDNA analyses and pushing the sensitivity of detection to the theoretical limits imposed by the plasma levels of ctDNA.*¹⁰²

100. Regarding Forshew (2012), Volik (2016) explains that Forshew (2012) had “relatively low sensitivity” for mutations below approximately 1%-2%. Volik (2016) summarizes Forshew (2012)’s approach as follows:

An early example of a PCR-based strategy for analysis of mutations found in individual patients involved the design of a set of tailed site-specific primers followed by multiplex PCR (for preamplification) and subsequent uniplex PCR using each individual primer pair. The second PCR, in which locus-specific primers are applied individually, is accomplished using a Fluidigm AccessArray system and the entire procedure was named TAm-Seq (Tagged Amplicon Sequencing; ref. 75).¹⁰³

This approach afforded the opportunity to quantify mutant DNA at many loci in each patient *but had a relatively low sensitivity for mutations below approximately 1%–2%.*¹⁰⁴

101. The lack of sensitivity of Forshew (2012)’s approach was significant because, among other things, the highly fragmented nature of cfDNA, and the low abundance of ctDNA in blood plasma, presented significant challenges in the biochemical preparation and analysis of cancer-associated genetic variants.

¹⁰² *Id.* at 906 (emphasis added).

¹⁰³ *Id.* at 903.

¹⁰⁴ *Id.* (emphasis added).

102. I also note that, in my experience, ultra-deep sequencing was not a common approach in 2014-2015.¹⁰⁵ Achieving this depth of read by sequencing tens or hundreds or thousands of times in the same region is typically unnecessary to achieve research goals. At the time, most researchers were not trying to find the “needle in the haystack” that this ultra-deep sequencing can accomplish. This approach would have been comparatively (and undesirably) expensive to the extent even available, which would have discouraged one of ordinary skill in the art from its use.

2) Ultra-Deep Sequencing Would Not Have Been An Obvious Modification of ForsheW (2012) In Light of the Error Rate of ForsheW (2012)’s Method

103. As noted *supra*, I disagree with Dr. Van Ness’s opinions that the ’454 Patent claims are obvious over ForsheW (2012).¹⁰⁶ The consequence of the sequencing workflow errors described *supra* is that, following ForsheW (2012)’s approach, rare genetic variants—*e.g.*, SNVs on tumor cfDNA present in the plasma at low levels—are not likely to be detectable below the “noise” of the error rate even if amplified 50,000 times or more at each locus. Dr. Van Ness does not address this problem, nor explain why a one of skill in the art would have been motivated to sequence to the claimed depth of read to remediate ForsheW (2012)’s limited sensitivity, or have reasonably expected success in doing so.

¹⁰⁵ See, *e.g.*, Volik (2016), discussed *supra* in Section II.

¹⁰⁶ See Van Ness Decl. at ¶¶ 142, 144.

104. Specifically, as a skilled artisan would have recognized, genetic variants in tumor-associated cfDNA are likely to be low-abundance or variable in patient plasma and consequently rare and difficult to target.¹⁰⁷ Forshew (2012) itself explicitly describes very similar challenges in working with cfDNA. For example, Forshew (2012) acknowledges that cfDNA is highly fragmented.

Circulating DNA is fragmented to an average length of 140 to 170 base pairs (bp) and is present in only a few thousand amplifiable copies per milliliter of blood, of which only a fraction may be diagnostically relevant (2, 3, 23–25). Recent advances in noninvasive prenatal diagnostics highlight the clinical potential of circulating DNA (25–28), but also *the challenges involved in analysis of circulating tumor DNA (ctDNA), where mutated loci and AFs may be more variable*. Various methods have been optimized to detect extremely rare alleles (1, 2, 6, 7, 29–31), and can assay for predefined or hotspot mutations. *These methods, however, interrogate individual or few loci and have limited ability to identify mutations in genes that lack mutation hotspots*, such as the TP53 and PTEN tumor suppressor genes (32). In patients with more advanced cancers, ctDNA can comprise as much as 1% to 10% or more of circulating DNA (2), presenting an opportunity for more extensive genomic analysis. Targeted resequencing has been recently used to identify mutations in selected genes at AFs as low as 5% (33–35). However, *identifying mutations across sizeable genomic regions spanning entire genes at an AF as low as 2%, or in few nanograms of fragmented template from circulating DNA, has been more challenging*.¹⁰⁸

105. It would have been necessary to detect and correct these sequencing workflow errors in order to sequence deeply enough to practice the claimed depth of read

¹⁰⁷ See Section II.

¹⁰⁸ See Forshew (2012) at 1 (emphases added).

in a plasma sample of cancer patient DNA. This is because SNVs associated with a particular cancer are present at relatively low levels in ctDNA, and, consequently, these sequencing workflow errors need to be detected and corrected to identify rare genetic variants at the targeted loci. Without such modifications, one of ordinary skill in the art would have understood that repeatedly sequencing a locus up to 50,000 times or more—which in any event was not taught or suggested by Forshew (2012)—would not have improved detection of rare genetic variants. Indeed, such repeated (and costly) sequencing would merely propagate the “noise” of the sequencing workflow errors without identifying the targeted rare genetic variant.

106. On this critical point, the Van Ness Declaration’s analysis is profoundly deficient. Dr. Van Ness fails to explain why one of ordinary skill in the art would have reasonably expected success in improving the sensitivity of Forshew (2012)’s method to detect rare variants specifically simply by increasing the sequencing depth of read to 50,000 or more per locus. In fact, doing so would not have remediated the deficiencies of Forshew (2012) because, as explained *supra*, performing deep sequencing to a read depth of 50,000 or more will simply increase the sequencing workflow noise. An substantial increase in the sequencing workflow noise would further obfuscate the detection of rare mutations, making it much harder to identify such mutations. In opining that it would have been obvious to increase the depth of sequencing read, Dr. Van Ness fails to account for any of these challenges that one of ordinary skill in the art would have confronted in 2014-2015.

107. Accordingly, contrary to the Van Ness Declaration’s contention¹⁰⁹, the parameter of sequencing read depth would *not* merely be “routinely adjusted to achieve whatever sensitivity was required.” In fact, it would have been nonsensical to undertake the time, effort, and expense of sequencing many thousands of times if doing so would not have been predicted to identify rare variants above the background “noise” (error rate) of the sequencing approach. A skilled artisan would not have been motivated to pursue this strategy, and would not have reasonably expected success doing so.

108. Other rationale provided in the Van Ness Declaration fails to support a conclusion that the ’454 Patent claims would have been obvious over Forshew (2012). The Van Ness Declaration’s reliance on the ’189 Patent Pub. (Bentley) reference¹¹⁰ does not cure the deficiencies in Dr. Van Ness’s rationale identified *supra*. The Van Ness Declaration relies on the ’189 Patent Pub. (Bentley) for the general proposition that increasing sequencing depth increases sensitivity,¹¹¹ but fails to explain how the ’189 Patent Pub. (Bentley) addresses the technical considerations I identified *supra*. To support the Van Ness Declaration’s proposition “that increasing sequencing depth increases sensitivity, Dr. Van Ness relies on Table 2 in the ’189 Patent Pub. (Bentley).¹¹² The Van

¹⁰⁹ See Van Ness Decl. at ¶ 143.

¹¹⁰ See Dkt. 118-9, U.S. Patent Application Publication No. US 2010/0261189) to Bentley *et al.* (“System and method for detection of HLA variants”) (the “’189 Patent Pub. (Bentley)”).

¹¹¹ See Van Ness Decl. at ¶ 144.

¹¹² *Id.*

Ness Declaration fails to appreciate that the '189 Patent Pub. (Bentley) is describing this relationship using error-free reads.

For example, based on binomial statistics the lower limit of detection (i.e., one event) for a fully loaded 60 mmx60 mm PicoTiterPlate (***2x10⁶ high quality bases, comprised of 200,000x100 base reads***) with 95% confidence, is for a population with allelic frequency of at least 0.002%, and with 99% confidence for a population with allelic frequency of at least 0.003% (it will also be appreciated that a 70x75 mm PicoTiterPlate could be employed as described above, which allows for an even greater number of reads and thus increased sensitivity).¹¹³

109. One of ordinary skill in the art would have understood that the “high quality bases” described in '189 Patent Pub. (Bentley) are error-free reads. As such, I do not see such a solution to the sequencing workflow errors described *supra* from my review of the '189 Patent Pub. (Bentley). Dr. Van Ness’s citation of prior testimony by Natera witnesses, including myself, also fails to account for the importance of sequencing workflow errors in the detection of rare variants for the same reason.¹¹⁴

110. Moreover, consistent with the Van Ness Declaration, Dr. Van Ness’s deposition testimony (excerpted *infra*) fails to address how one of ordinary skill in the art would have overcome the challenges Forshew (2012) presents. Dr. Van Ness opined that the NGS platforms are “very accurate,” which is an oversimplification of the technology, particularly because—as explained *infra*—the Examiner distinguished Forshew (2012)

¹¹³ See '189 Patent Pub. (Bentley) at [0085] (emphasis added).

¹¹⁴ See Van Ness Decl. at ¶¶ 145-150.

from the '454 Patent claims on the basis of sensitivity. And in deposition, Dr. Van Ness did not explain how the conventional NGS technology of Forshew (2012) could be used to identify rare variants that occur below these platforms' known error rate. Dr. Van Ness's testimony merely confirms that more information is obtained with each read—but, given the error rate, it would have been challenging if not impossible to determine or distinguish between information arising from accurately sequencing a rare genetic variant and information arising from an error in the preparation or sequencing process.

Q. Is... Are you aware of any problems that occur -- technical problems that occur by increasing depth of read?

A. You know, anytime you do an experiment and you extend the experiment, you have to weigh the pluses and minuses. *So I think, are there opportunities for errors that increase with sequencing depth?* Maybe. But you get more information. So there's much more benefit and enhanced sensitivity particularly because the platforms are very accurate. And so I think with the Illumina platforms -- I mean, the whole reason for doing 50,000 is because you could have an error of 5 or 6 base calls, but because you did 50,000, you're -- you're looking at a very large sampling so that you are more and more assured that the sequences that you're getting are correct.¹¹⁵

111. In sum, Forshew (2012) identifies a significant problem—the desirability of identifying of rare genetic variants in tumor-associated cfDNA—without providing any solution. It would not have been obvious to remediate this deficiency by combining the

¹¹⁵ See Van Ness Tr. at 102:2-103:14, specifically citing 102:2-21 (objection omitted, emphasis added).

claimed depth of read with Forshew (2012)’s method for reasons explained *supra*. Dr. Van Ness’s analysis does not support a different conclusion because the Van Ness Declaration does not articulate how one of ordinary skill in the art would have reasonably expected to use sequencing to “a depth of read of at least 50,000 per target locus” effectively to identify rare mutations in light of the known limitations of the available equipment and approaches. Moreover, as discussed further *infra*, Forshew (2012) does not identify a specific technique that will work for this purpose.

112. The lack of an obvious solution is further reflected in the Editor’s Summary accompanying Forshew (2012)’s publication. The Summary notes (emphasis added) that “TAm-Seq ***will need to achieve a more sensitive detection limit*** (<2% allele frequency) to identify mutations in the plasma of patients with less advanced cancers”¹¹⁶—*e.g.*, patients with less tumor-associated cfDNA present in plasma and, consequently, more rare tumor-associated genetic variations. The Editor’s Summary continues that “***once optimized***, this ‘liquid biopsy’ approach will be amenable to personalized genomics.”¹¹⁷ Accordingly, the Editor’s Summary is further evidence of how one of ordinary skill in the art would not have reasonably expected success in achieving the claimed methods following the teachings of Forshew (2012) based on the approach identified in the Van Ness Declaration.

¹¹⁶ See NAT-NEO-00043262-00043275 (Editor’s Summary to Forshew (2012)) at NAT-NEO-00043262 (emphasis added).

¹¹⁷ *Id.*

3) ForsheW (2012) Does Not Teach that Ultra-Deep Sequencing Would Have Improved the Sensitivity of Its Approach

113. The Van Ness Declaration suggests ForsheW (2012) itself teaches sequencing more deeply to achieve the methods claimed in the '454 Patent.¹¹⁸ I disagree that ForsheW (2012) proposed possibilities that could be tried to improve the sensitivity of detection of TAm-Seq.

114. First, ForsheW (2012) specifically would not have motivated one of ordinary skill in the art to achieve the claimed read depth because ForsheW (2012) was not trying to find a rare genetic variant at a sensitivity of 0.1%, and ForsheW (2012) does not teach that this particular goal would have been desirable to achieve.

115. Second, ForsheW (2012) would not have motivated one of ordinary skill in the art to achieve the claimed read depth because it does not teach that this solution would remediate the admitted limitations of ForsheW (2012)'s approach. At page 10 (reproduced *infra*), ForsheW (2012) merely references "higher read depth" in the context of four other suggestions—higher fidelity, additional replicates, improve algorithms, and rare allele enrichment. None of these suggest increasing the read depth to the claimed levels.

Higher read depth or fidelity, additional replicates, or improved algorithms **could allow** for enhanced mutation detection without change to protocols. An alternative strategy is through rare allele enrichment, for example, by combining

¹¹⁸ See Van Ness Decl. at ¶ 142.

TAm-Seq with protocols such as COLD-PCR (co-amplification at lower denaturation temperature PCR).¹¹⁹

116. Notably, Forshew (2012) does not state that any of these strategies, including higher read depth, would necessarily solve its issues with detecting rare mutations, only that they “could”—hypothetically—“allow for enhanced mutation detection.” Forshew (2012) thus speculates about what other approaches “could allow” rather than teach (much less test) how to address its shortcomings.

117. Consistent with my opinions, Dr. Van Ness acknowledged that there were “challenges” in Forshew (2012)’s strategy.

Q. In discussing targeted resequencing, Dr. Forshew says that, quote, “However, identifying mutations across sizable genomic regions spanning entire genes at an AF as low as 2%, or in few nanograms of fragmented template from circulating DNA, has been more challenging.” So that – so according to Dr. Forshew, the targeted resequencing has not solved that particular challenge, right?

A. *Again, it’s more challenging.* Didn’t say it wasn’t doable. He just said it’s more challenging.

Q. Okay. And do you agree with him? Or disagree with him?

A. *No, I agree that at the time he was sort of justifying his publication, that he recognized that there were challenges,* not impossibilities, but there were challenges that could bear improvement.¹²⁰

¹¹⁹ See Forshew (2012) at 10 (emphases added). The underlined portions represent the other possibilities suggested by Forshew (2012) that the Van Ness Decl. failed to disclose.

¹²⁰ See Van Ness Tr. at 120:20-121:16 (objection and stricken question omitted, emphases added).

118. I am informed and understand from counsel that an invention is not obvious merely because there are a finite number of options available for one of ordinary skill in the art to pursue—rather, the skilled artisan must also have had a reasonable expectation of success that a particular solution will work. Here, one of ordinary skill in the art would not have reasonably expected success with any particular idea proposed by Forshew (2012) *supra*, including increasing sequencing read depth. In fact, Forshew (2012)’s speculative suggestion of different possible approaches, all stated generically without any implementation details, and with no further reason given to select one or another, indicates that Forshew (2012) did not know which approach, if any, would have been effective to improve the sensitivity of the disclosed TAm-Seq approach.

C) ForsheW (2012) Was Overcome During Prosecution

119. I understand that Forshew (2012) was before the Examiner during prosecution of the ’454 Patent. I have been informed and understand from counsel that, when prior art is before the Examiner, the Examiner is presumed to have properly considered the prior art. I have reviewed the ’454 Patent application’s prosecution history and I see no basis to believe that the Examiner misunderstood the teachings of Forshew (2012) or other teachings or knowledge in the art, or otherwise erred in allowing the ’454 Patent claims.

120. As the Van Ness Declaration acknowledges, the Examiner of the '454 Patent application actively considered Forshew (2012) during prosecution of the '454 Patent application and determined that the pending claims were allowable over Forshew (2012).¹²¹

121. The Van Ness Declaration nevertheless substitutes Dr. Van Ness's judgment for that of the Examiner and asserts that the Examiner accepted "erroneous arguments" by Natera in allowing the '454 Patent claims over Forshew (2012).¹²² I disagree. As excerpted from Forshew (2012) *infra*, Forshew (2012) teaches, among other things, the use of targeted single-plex amplification, as Natera rightly pointed out, and Natera accurately quotes Forshew (2012) regarding potential problems associated with multiplexing.¹²³ There is no evidence that the Examiner did not independently consider the entirety of Forshew (2012)'s disclosure in finding the '454 Patent claims patentable over its teachings.

Performing single-plex amplification with each of these primer pairs would require dispersing the initial sample into many separate reactions, considerably increasing the probability of sampling errors and allelic loss. ***Multiplex amplification using a large set of primers could result in nonspecific amplification products and biased coverage.*** We therefore applied a two-step amplification process: a limited-cycle preamplification step where all primer sets were used together to capture the starting molecules present in the template, ***followed by individual amplification to purify and select for intended targets*** (Fig. 1B) (Supplementary Methods).¹²⁴

¹²¹ See Van Ness Decl. at ¶¶ 166-176.

¹²² *Id.* at ¶¶ 168-169; *see also* Dkt. 118-10 (Remarks to an Office Action Dated October 7, 2022) & Dkt. 118-11 (Notice of Allowance Dated November 8, 2022).

¹²³ *Id.* at ¶ 168.

¹²⁴ See Forshew (2012) at 3 (emphases added),

122. Moreover, the '454 Patent claims recite more than just multiplex amplification—for example, as discussed *supra* they recite an ultra-deep depth of read of 50,000 or more, which it is undisputed Forsheew (2012) does not disclose. The Examiner specifically pointed to this limitation—*not* multiplex amplification—as a basis for allowing the claims. Specifically, on page 5 of the '454 Patent application's notice of allowance¹²⁵, the Examiner stated:

Reasons for Allowance

19. The following is an examiner's statement of reasons for allowance: The prior art does not show the limitation in independent claim 1 of a sequencing read depth of at least 50,000 per target locus in the context of the claimed subject matter. The prior art does not show the limitation in independent claim 15 of detecting a single nucleotide variant mutation that is present in less than or equal to 0.015% of the cell-free DNA comprising the single nucleotide variant locus in the context of the claimed subject matter.

123. The Van Ness Declaration contends, however, that it “seems likely” that the Examiner overlooked the obviousness of increasing sequencing depth.¹²⁶ This contention is contradicted by Dr. Van Ness's assertion that increasing sequencing depth was a well-known approach to improve sensitivity, as evidenced (and Dr. Van Ness contends) by the '189 Patent Pub. (Bentley).¹²⁷

¹²⁵ See Dkt. 118-11 (Notice of Allowance Dated November 8, 2022) at 5.

¹²⁶ See Van Ness Decl. at ¶ 144.

¹²⁷ *Id.* at ¶¶ 170-175.

124. Additionally, the Van Ness Declaration identifies no factual basis to indicate that the Examiner did not consider all claim limitations, including sequencing to a read depth of 50,000 or more per target locus. Indeed, if anything, it seems unlikely that the Examiner would not have considered the arguments by Natera given that Forshew (2012) was explicitly identified during prosecution by the Examiner and then subsequently addressed in substance by Natera. In addition, Dr. Van Ness does not address the points I raise *supra*—namely, that sequencing to the claimed read depth would not have been obvious because one of ordinary skill in the art would not have expected or predicted this strategy to be effective to identify rare variants when using Forshew (2012)’s approach. Thus, the allowance of the claims is consistent with my opinions that such a sequencing strategy would not have been obvious to one of ordinary skill in the art.¹²⁸

(ii) *Bashashati (2013) Does Not Render the ’454 Patent Claims Obvious*

125. At least for the reasons explained *infra*, the Van Ness Declaration has failed to show that the ’454 Patent claims are obvious over Bashashati (2013).¹²⁹ As described *infra*, I provide my reasons and bases for why I disagree with Dr. Van Ness’s opinions regarding Bashashati (2013).¹³⁰

¹²⁸ See Dkt. 118-11 (Notice of Allowance Dated November 8, 2022).

¹²⁹ See Dkts. 118-14, 118-15, Bashashati *et al.*, *Distinct evolutionary trajectories of primary high-grade serous ovarian cancers revealed through spatial mutational profiling*, JOURNAL OF PATHOLOGY 231:21-34 (2013), including the Supplementary Appendix (“Bashashati (2013)”).

¹³⁰ Dr. Van Ness admitted in deposition that he did not include a specific section in the Van Ness Declaration analyzing the combination of Forshew (2012) and Bashashati (2013). See Van Ness Tr. at 106:7-13.

126. For the claims to be obvious, *inter alia*, I have been informed and understand that it must have been obvious to one of ordinary skill in the art to perform “targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume” in the course of performing the other method steps. Bashashati (2013) does not perform this approach, as Dr. Van Ness acknowledges,¹³¹ and my analysis here focuses primarily on ’454 Patent Claim 1[b].

127. Dr. Van Ness’s contrary opinions conflate disparate teachings in Bashashati (2013) without articulating how one of ordinary skill in the art would have been motivated to practice the claimed methods or would have reasonably expected success in doing so, particularly given the difficulties in multiplexing many target loci in a single reaction volume. The results of this multiplexing strategy would have been unpredictable in light of the technical challenges in its implementation, as discussed further *infra*. I reserve the right to offer additional opinions regarding the ’454 Patent in view of Bashashati (2013) alone or in combination with other references.

(a) Claim 1[b] of the ’454 Patent

performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a

¹³¹ See, e.g., Van Ness Decl. at ¶ 190.

plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume

128. The '454 Patent claims require “targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume.”¹³² I refer to this limitation as claim 1[b] of the '454 Patent.

129. The Van Ness Declaration does not opine that Bashashati (2013) teaches this approach. Instead, in arguing this limitation is obvious, Dr. Van Ness points to Bashashati (2013)’s description of using target-specific primers to amplify tumor DNA and then using those same primers to amplify ctDNA.¹³³ For both Bashashati (2013)’s tumor and ctDNA samples, however, individual primer pairs were used, not multiplex PCR as required by Claim 1 of the '454 Patent.

130. Regarding the tumor DNA analysis, Bashashati (2013) explains that “DNA primers were synthesized in 96-well plates ... Polymerase cycling reactions were set up in 96-well plates and comprised of 0.5 uM forward primer, 0.5 uM reverse primer, 1-2 ng of gDNA... Reaction plates were cycled ... PCR reactions were visualized on 3% agarose

¹³² See '454 Patent at 171:32-38.

¹³³ See, e.g., Van Ness Decl. at ¶ 189.

... Successful reactions were manually pooled.”¹³⁴ Similarly, regarding the ctDNA analysis, Bashashati (2013) explains that “[t]he primers used and PCR cycling conditions were the same as in the previous section. Circulating DNA was used as PCR templates at 0.1 ng per reaction.”¹³⁵

131. From these descriptions, the Van Ness Declaration can only say that “[t]hese sections teach the use of targeted PCR to amplify target loci encompassing different SNV.”¹³⁶ Dr. Van Ness cannot and does not say that Bashashati (2013) teaches targeted multiplex PCR on ctDNA from plasma samples.

132. Indeed, the Van Ness Declaration acknowledges that in Bashashati (2013), “targeted amplification of SVNs from ctDNA was conducted using *single primer pairs per reaction*.”¹³⁷ I agree with the Van Ness Declaration’s implicit acknowledgement that Bashashati (2013) does not disclose claim 1[b] of the ’454 Patent. Dr. Van Ness in deposition agreed that Bashashati (2013) does not teach targeted multiplex amplification of 10 to up to 500 targets in one reaction volume.¹³⁸

133. To argue the ’454 Patent claims are obvious, the Van Ness Declaration attempts to cure this deficiency in Bashashati (2013)’s disclosure in four ways, discussed

¹³⁴ See Bashashati (2013) at S6-S7.

¹³⁵ *Id.* at S7.

¹³⁶ See Van Ness Decl. at ¶ 190.

¹³⁷ *Id.* at ¶ 195 (emphasis added).

¹³⁸ See Van Ness Tr. at 134:2-13, particularly 134:11-13 (“I don’t know of an example of that, although there are other references that I referred to that have done it. This one I have not seen it.”).

in turn *infra*. None of these points are successful because Dr. Van Ness fails to explain how one of ordinary skill in the art would have been motivated to modify Bashashati (2013), much less with any reasonable expectation of success.

134. First, Dr. Van Ness states in a single, unsupported sentence that a “POSA also would have been motivated to do so [amplifying target loci in multiplex reactions in the same reaction volume] for the benefit of requiring fewer separate reactions.”¹³⁹ I disagree. Dr. Van Ness identifies no actual or theoretical benefit that fewer separate reactions would confer in the performance of the methods taught in Bashashati (2013). In fact, combining multiple PCR-based amplification reactions—especially up to 150 such reactions—in one volume was associated with known significant technical hurdles and, consequently, would have been an undesirable strategy for one of ordinary skill in the art to have pursued in the context of the claimed methods.

135. The ’454 Patent itself explains some of those technical hurdles. In light of these challenges, the outcome of multiplexing many primer pairs in a single reaction volume would have been unpredictable to a skilled artisan.

[H]ighly multiplexed PCR can often result in the production of *a very high proportion of product DNA that results from unproductive side reactions such as primer dimer formation*. In an embodiment, the particular primers that are most likely to cause unproductive side reactions may be removed from the primer library to give a primer library that will result in a greater proportion of amplified DNA that maps to the genome.¹⁴⁰

¹³⁹ *Id.*

¹⁴⁰ *See* ’454 Patent at 105:51-58 (emphasis added).

Multiplexing large numbers of primers imposes considerable constraint on the assays that can be included. Assays that unintentionally interact result in spurious amplification products.¹⁴¹

Such challenges were known even earlier, as disclosed in Natera's '035 Patent.¹⁴² One of ordinary skill in the art would not have reasonably expected success in increasing the multiplexing of any of Bashashati (2013)'s PCR reactions in a single volume. The Van Ness Declaration offers no basis to believe otherwise. Rather, one of ordinary skill in the art would have viewed consolidating multiple reactions into a single volume as potentially introducing complications to the reactions, as opposed to facilitating them. Accordingly, it would not have been obvious to one of ordinary skill in the art to perform the claimed targeted multiplex amplification of tumor-associated cfDNA in a single read volume.

136. Second, the Van Ness Declaration argues that one of ordinary skill in the art could have looked to a different example in Bashashati (2013) that used the Ion Torrent PGM platform to perform targeted multiplex PCR to validate SNVs.¹⁴³ I disagree that this is support for obviousness for several reasons. The Ion Torrent example was performed on tumor tissue samples, not on ctDNA isolated from plasma.¹⁴⁴ Indeed, the portion of

¹⁴¹ *Id.* at 107:65-108:1 (emphasis added).

¹⁴² *See, e.g.*, '035 Patent at 48:7-14, 54:26-29.

¹⁴³ *See* Van Ness Decl. at ¶ 195.

¹⁴⁴ *See* Bashashati (2013) at 25. "Forty-three mutations present in the right ovary (mixed histology) were not present in the samples from the left (all HGSCs; Figure 1 B [case 4]). As the index exome for [case 4] was restricted to the right set of samples, we subjected all samples to the PGM Ion AmpliSeq Cancer panel."

Bashashati (2013) quoted in the Van Ness Declaration¹⁴⁵ asserts that Bashashati (2013) performed the PGM Ion AmpliSeq Cancer panel on “primary ovarian cancer samples.”

137. Furthermore, the Van Ness Declaration fails to explain why one of ordinary skill in the art would have been motivated to use the Ion Torrent-based approach, as previously applied merely for the purpose of validating candidate SNVs in DNA from tumor samples. In tumor samples, in tumor-associated DNA is not rare. The Van Ness Declaration fails to explain why the use of tumor tissue samples would render obvious the claimed methods using cfDNA. As explained *supra*,¹⁴⁶ working with cfDNA from plasma samples was challenging and unpredictable for many reasons, such as cfDNA fragmentation. Identifying genetic mutations in tumor-associated cfDNA would have been even more difficult, given that tumor-associated cfDNA is relatively low-abundance and highly variable within a person’s cfDNA. The Van Ness Declaration also fails to explain how one of ordinary skill in the art would have reasonably expected success with the Ion Torrent-based approach in light of these technical challenges. Accordingly, it would not have been obvious to use a method used only for validating SNVs in tissue-derived DNA in order to perform the claimed targeted multiplexing of cfDNA in a single reaction volume.

138. Third, the Van Ness Declaration points to Forsheo (2012) because Dr. Van Ness contends Bashashati (2013) cites Forsheo (2012) as support for the proposition that

¹⁴⁵ See Van Ness Decl. at ¶ 195.

¹⁴⁶ See *supra* at Section II.

“sequencing of cell-free circulating tumour DNA (ctDNA) extracted from plasma has been demonstrated to be an effective non-invasive tool for monitoring tumour burden.”¹⁴⁷ This citation, however, is not relevant to obviousness here because it says nothing relating to the claimed targeted multiplex amplification in a single reaction volume. In fact, this citation indicates that Bashashati (2013) was aware of Forshew (2012) but ***chose not to follow*** Forshew (2012)’s approach to the extent Forshew (2012) teaches multiplex amplification of multiple loci in a single reaction volume.

139. Consistent with my opinions, Forshew (2012) recognized the technical challenges associated with multiplexing many reactions in a single volume. Forshew (2012) explains that “[m]ultiplex amplification using a large set of primers could result in nonspecific amplification products and biased coverage.”¹⁴⁸ As a consequence of those admitted technical challenges, the Forshew (2012) strategy required a singleton amplification step, which one of ordinary skill in the art would have recognized from reading Forshew (2012). In deposition, Dr. Van Ness confirmed that Forshew (2012)’s strategy did not involve multiplexing independent of single-plex amplification. Dr. Van Ness stated that “Dr. Forshew incorporated the multiplex reaction in his series of steps. I didn’t see anywhere where he did just the multiplex and analyzed it. But he incorporated it into all of his steps.”¹⁴⁹ Thus, these limitations in Forshew (2012) would have further

¹⁴⁷ See Van Ness Decl. at ¶ 196.

¹⁴⁸ See Forshew (2012) at 3 (emphasis added).

¹⁴⁹ See Van Ness Tr. at 129:24-130:11 (objection omitted).

discouraged one of ordinary skill in the art from modifying Bashashati (2013) to perform targeted multiplex amplification of cfDNA in a single volume.

140. Fourth, the Van Ness Declaration contends that the “ability to use greater multiplex targeted amplification would also have been obvious to one of ordinary skill in the art prior to the priority date for the claim because even significantly greater multiplex amplification of DNA was already well established.”¹⁵⁰ I disagree. The teachings that Dr. Van Ness relies on do not teach greater multiplexing in a single reaction volume. In fact, they teach the opposite, particularly in light of known challenges in multiplexing as discussed *supra*.¹⁵¹

141. For example, the Van Ness Declaration relies on the '038 Patent Pub. (Mir) (*i.e.*, Fluidigm system).¹⁵² Similar to Forsheo (2012), Fluidigm uses a preamplification step using multiplex PCR followed by singleton PCR (each reaction in its own reaction volume) to generate individual target-specific amplicons.¹⁵³ In fact, Forsheo (2012) uses

¹⁵⁰ See Van Ness Decl. at ¶¶ 197-199.

¹⁵¹ See, *e.g.*, '454 Patent at 105:51-58, 107:65-108:1.

¹⁵² See Dkt. 116-13, Mir et al., *Assay methods for increased throughput of samples and/or targets*, U.S. Patent Application Publication No. US 2010/0120038 (2010) (the “'038 Patent Pub. (Mir)”).

¹⁵³ See '038 Patent Pub. (Mir) at [0119] (“In illustrative embodiments, the encoding reaction can be a preamplification reaction, which may be carried out on a microfluidic device. To increase target nucleic acid concentration prior to encoding, an optional pre-preamplification reaction can be carried out before the encoding preamplification reaction. The pre-preamplification can be carried out in multiplex [*sic*]. For example, target-specific primers for 9216 different target nucleic acids can be employed in one mixture. This mixture can then be divided into R=96 aliquots and each aliquot subjected to an encoding preamplification reaction on a microfluidic device, using T=96 different primer pairs that

the Fluidigm system in its singleton PCR step.¹⁵⁴ Dr. Van Ness's citation to a previous, unrelated declaration I submitted in an *inter partes* review¹⁵⁵ ignores the context I provided, which explains the preamplification reaction is then aliquoted into individual wells to perform singleton PCR,¹⁵⁶ just as that described in Forsheew (2012).

142. As explained *supra*, Dr. Van Ness fails to show that one of ordinary skill in the art would have been motivated to modify Bashashati (2013)'s approach to perform "targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume," or that one of ordinary skill in the art would have reasonably expected to succeed in doing so given

add 96 different nucleotide tag pairs to the target nucleotide sequences in each of the 96 aliquots."); *id.* at [0120] ("After the encoding preamplification reaction, amplification can be carried out in separate chambers of a microfluidic device. For example, each of the 96 aliquots produced upon encoding preamplification can be loaded into individual sample lines of a matrix-type microfluidic device, and each of 96 different tag-specific primer combinations can be loaded into individual assay columns. Each different of the 96 primer combination can amplify a different target nucleic acid in each of the 96 aliquots. The resulting 9216 reaction chambers (sub-aliquots) can then be subjected to amplification, followed by detection of amplification product(s), which can be carried out by any suitable means, including SYBR Green, universal probe library, use of one probe per tag combination (e.g., wherein probe sequences are introduced into nucleotide tags), use of fluorescent primers to add nucleotide tags.").

¹⁵⁴ See Forsheew (2012) at S3 ("Individual primer pairs were loaded into the primer inlets of the Access Array IFC (Fluidigm).").

¹⁵⁵ See Dkt. 116-12, *Declaration of Michael L. Metzker, Ph.D.*; IPR2018-01317, dated June 29, 2018 ("Metzker IPR Decl.").

¹⁵⁶ *Id.* at ¶ 100.

the well-known technical difficulties in multiplexing many targets in one reaction volume, as recognized in Forshew (2012), for example.

143. Consistent with my opinions, Dr. Van Ness admitted in deposition that multiplexing many targets in one reaction volume could lead to undesirable consequences.

Q. What kinds of challenges are involved in amplifying multiple targets together in one reaction volume that are not challenges in amplifying multiple targets individually in separate reaction volumes?

A. *Primarily, I think one of the challenges is primer interactions.* Because you have multiple primers, you have *potential for primer interactions that could interfere with the amplification of the targets* you're after.

Q. Is another problem – or another challenge – allelic bias?

A. *It can be.*¹⁵⁷

144. I disagree with Dr. Van Ness's evaluation of Bashashati (2013) and the opinions expressed in the Van Ness Declaration on the '454 Patent claims' obviousness in other respects. For example, the Van Ness Declaration asserts that the use of an amplicon length of 50-150 base pairs "would have been obvious, particularly in the context of cell-free DNA."¹⁵⁸ I disagree. The Van Ness Declaration provides no reasons or bases to support this opinion. In fact, Bashashati (2013) uses Illumina's MiSeq system to sequence the amplicons from ctDNA isolated from plasma using "paired-end 250 bp [base pair]

¹⁵⁷ See Van Ness Tr. at 132:13-133:3 (emphases added).

¹⁵⁸ See Van Ness Decl. at ¶¶ 192-193.

reads.”¹⁵⁹ These sequence reads are anywhere from 100 to 200 bp longer than amplicon length on which Dr. Van Ness opines. The Van Ness Declaration provides no explanation why one of ordinary skill in the art would have deviated from a sequencing system that provided 250 base pair reads to achieve an amplicon length of 50-150 bases. Bashashati (2013)’s use of MiSeq would in fact inform the one of ordinary skill in the art that the amplicon lengths would have been closer to 250 base pairs to maximize the performance of the Illumina MiSeq system.¹⁶⁰

(b) Claim 1[c] of the ’454 Patent

sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads, wherein the sequencing has a depth of read of at least 50,000 per target locus

145. The Van Ness Declaration’s opinions regarding the ’454 Patent’s sequencing step are also deficient or factually incorrect. Dr. Van Ness asserts, for example, that “Bashashati [] teaches sequencing amplicons on an Illumina GAI.”¹⁶¹ But Bashashati (2013) uses the GAI to sequence amplicons from “tumour and normal DNA templates,”¹⁶² not ctDNA obtained from plasma samples.

¹⁵⁹ See Bashashati (2013) at S7.

¹⁶⁰ *Id.* at S6.

¹⁶¹ See Van Ness Decl. at ¶ 201.

¹⁶² See Bashashati (2013) at S6.

146. The Van Ness Declaration also fails to establish that one of ordinary skill in the art would have had a reasonable expectation of success in achieving the methods claimed in the '454 Patent, *e.g.*, to the extent one of ordinary skill in the art would have been motivated to perform ultra-deep sequencing to “a depth of read of at least 50,000 per target locus.” I discuss deficiencies in Dr. Van Ness’s reasoning *infra*.

147. First, as explained *supra* with respect to Forshew (2012), as of 2014-2015, ultra-deep sequencing would not have been useful to identify rare genetic variants which are buried in the “noise” of sequencing workflow errors associated with sample preparation and next-generation sequencing technology. Dr. Van Ness fails to account for these technical limitations in his assessment of Bashashati (2013). For example, one of ordinary skill in the art would have understood that the 454 Life Sciences’ PicoTiterPlate format, used in Bashashati (2013), has an error rate of ~1%.¹⁶³ As the error rate dictates the frequency at which SNVs can be identified, Table 2 in the '189 Patent Pub. (Bentley) (cited by Dr. Van Ness) suggests that read depths between 400 and 1,000 would have been sufficient to identify an SNP at the 1% level, as shown *infra* (red box added to highlight the number of reads to an SNP at ~1% level).

¹⁶³ See Ex. 7, Gilles *et al.*, *Accuracy and quality assessment of 454 GS-FLX Titanium pyrosequencing*, BMC GENOMICS 12:245 pp. 1-11 (2011) (“Gilles (2011)”) at Abstract: “We obtained a mean error rate for 454 sequences of 1.07%.”

TABLE 2

SNP Classes	Number of Reads	Minimum frequency of SNP in population detectable with 95% confidence	Minimum frequency of SNP in population detectable with 99% confidence
1	200000	0.002%	0.003%
2	100000	0.005%	0.007%
5	40000	0.014%	0.018%
10	20000	0.028%	0.037%
50	4000	0.14%	0.18%
100	2000	0.28%	0.37%
200	1000	0.55%	0.74%
500	400	1.39%	1.85%
1000	200	2.76%	3.64%

Higher read depth would result in the detection of noise (*i.e.*, sequencing workflow errors).

148. Thus, in light of known sequencing error rates for all sequencing platforms discussed *supra*, I disagree with Dr. Van Ness that “sequencing depth is a parameter that would be routinely adjusted to achieve whatever sensitivity was required.”¹⁶⁴ In reality, some sensitivity levels would not have been achievable due to technical limitations of equipment or experimental design (*e.g.*, sample preparation), no matter how much sequencing depth was increased because of fundamental issues (*e.g.*, noise levels) with the system.

149. Second, and relatedly, the Van Ness Declaration points to the ’454 Patent’s disclosure for the propositions that “the ’454 patent does not identify any particular sequencing depth as being inventive or special” and “sequencing depth is a parameter that

¹⁶⁴ See Van Ness Decl. at ¶ 208.

would be routinely adjusted to achieve whatever sensitivity was required.”¹⁶⁵ I disagree with this reading of the patent and the premise. As explained *supra*, simply repeatedly sequencing a target locus will not improve the sensitivity of detection beyond the method’s inherent error rate and will not identify rare variants that fall below that threshold. In fact, the ’454 Patent describes the advantages of its inventive methods for this purpose.

In another aspect, the present invention generally relates, at least in part, to improved methods of detecting single nucleotide variations (SNVs). These improved methods include improved analytical methods, improved bioassay methods, and improved methods that use a combination of improved analytical and bioassay methods. ***The methods in certain illustrative embodiments are used to detect, diagnose, monitor, or stage cancer, for example in samples where the SNV is present at very low concentrations***, for example less than 10%, 5%, 4%, 3%, 2.5%, 2%, 1%, 0.5%, 0.25%, or 0.1% relative to the total number of normal copies of the SNV locus, such as circulating free DNA samples. That is, ***these methods in certain illustrative embodiments are particularly well suited for samples where there is a relatively low percentage of a mutation or variant relative to the normal polymorphic alleles present for that genetic loci***.¹⁶⁶

150. The Van Ness Declaration’s citations to my and others’ testimony¹⁶⁷ likewise fail to explain how one of ordinary skill in the art would have reasonably expected success in overcoming the technical challenges by modifying the Bashashati (2013) approach as explained *supra*. Dr. Van Ness also fails to explain why one of ordinary skill in the art

¹⁶⁵ *Id.* at ¶ 209.

¹⁶⁶ See ’454 Patent at 44:6-21 (under “Detailed Description of the Invention”) (emphases added).

¹⁶⁷ See Van Ness Decl. at ¶¶ 210-215.

would have been motivated to do so with respect to Bashashati (2013), and those citations are therefore irrelevant.

151. Third, the Van Ness Declaration also fails to show that Bashashati (2013) achieves the claimed sequencing depth of read as Dr. Van Ness misreads Bashashati (2013). In paragraphs 204-205 of the Van Ness Declaration, Dr. Van Ness identifies 17 rows from Table S2 of Bashashati (2013) (“lines 48, 54, 82, 91, 98, 111, 121, 124, 129, 130, 144, 151-153, 156, 170, and 173”) as support for the proposition that “[a] POSA would understand that these positive resequencing read values for tumor-specific SNVs indicate that Bashashati detected tumor-specific SNV mutations present in the cell-free DNA from the sequence reads.”¹⁶⁸ But none of these rows support his assertion and I therefore disagree. Dr. Van Ness is not reading Bashashati (2013) Table S2 correctly.

152. In particular, Column V of Bashashati (2013) (*i.e.*, Plasma BinomExact_adjustedpVal) is used to perform “detecting one or more of the tumor-specific SNV mutations.” Bashashati (2013) explains that “[t]he number of mutations based on positions with coverage (c), no coverage (nc) and those with coverage that were significantly (s) detected, based on the binomial extract test (adjusted $p < 0.05$).”¹⁶⁹ In contrast, for the rows cited *supra* in the Van Ness Declaration, only two rows, 98 and 156 have p values of < 0.05 —namely, 1.36×10^{-7} and 4.04×10^{-7} , respectively. The total number of reads for rows 98 and 156 are well below “a depth of read of at least 50,000 per

¹⁶⁸ *Id.* at ¶ 205.

¹⁶⁹ *See* Bashashati (2013) at Fig. 2 legend.

target loci.” For example, the “refcountplasma” (column T) and “altcountplasma” (column U) for row 98 is 8,190 and 105, which totals 8,295 reads. The “refcountplasma” (column T) and “altcountplasma” (column U) for row 156 is 78 and 5, which totals 83 reads. None of this comes anywhere close to the recited “of at least 50,000 per target locus.”

153. Dr. Van Ness also argues that “Bashashati teaches that the sequencing has a depth of read of at least 50,000” by pointing to row 276 showing a value of 969,361 in the “refcountplasma” (column T).¹⁷⁰ I disagree as, again, Dr. Van Ness is misreading Table S2 and again fails to demonstrate that Bashashati (2013) discloses this limitation. Column V (*i.e.*, Plasma BinomExact_adjustedpVal) of row 276 has a $p = 1$, which would fail the binomial extract test and therefore not have been identified as a SNV mutation.

B. The '035 Patent Is Not Obvious

(i) *Kaper (2010) Poster Does Not Render the '035 Patent Claims Obvious*

154. At least for the reasons explained *infra*, Dr. Van Ness has failed to show that the '035 Patent claims are obvious over the teachings of the Kaper (2010) Poster.¹⁷¹ I disagree with Dr. Van Ness’s opinions regarding the Kaper (2010) Poster.

¹⁷⁰ See Van Ness Decl. at ¶ 207.

¹⁷¹ See Dkt. 116-15, Kaper *et al.*, *Parallel preparation of targeted resequencing libraries from 480 genomic regions using multiplex PCR on the Access Array system*, PROCEEDINGS OF THE 101ST ANNUAL MEETING OF THE AMERICAN ASSOCIATION FOR CANCER RESEARCH (AACR), Poster (2010) (“Kaper (2010) Poster”).

155. For the claims to be obvious, *inter alia*, I have been informed and understand it must have been obvious to one of ordinary skill in the art the following claim elements in the course of performing the other method steps:

- Claim 1[a]: using “isolated cell-free DNA” that “is isolated from a blood sample”
- Claim 1[b]: to perform “targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume”
- Claim 1[c]: to perform “sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products, wherein the plurality of SNP loci comprises 25-2,000 loci associated with cancer”

Dr. Van Ness’s opinions regarding these individual limitations, separately and in combination, are deficient because nothing in the Kaper (2010) Poster would have motivated one of ordinary skill in the art to pursue the claimed combination. Dr. Van Ness’s opinions regarding these individual limitations, separately and in combination, are deficient because the skilled artisan would not have reasonably expected success in modifying Kaper to achieve them as Dr. Van Ness proposes, at least in light of the unpredictability and technical challenges in the art involved in manipulating cfDNA.¹⁷² I reserve the right to offer additional opinions regarding the ’035 Patent in view of the Kaper (2010) Poster alone or in combination with other references.

¹⁷² See Section II.A, *supra*.

(a) Claim 1[a] of the '035 Patent

tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products, wherein the isolated cell-free DNA is isolated from a blood sample collected from a subject who is not a pregnant women

156. The Van Ness Declaration asserts that “Kaper renders this claim limitation obvious.”¹⁷³ I disagree.¹⁷⁴ As a preliminary matter, the Kaper (2010) Poster does not teach or suggest amplifying or sequencing cfDNA. Rather, the samples used in Kaper (2010) Poster are obtained from tumor tissue samples or control (healthy) DNA from a HapMap sample (human genome DNA isolated from a cell line). The Van Ness Declaration does not contend otherwise.

157. Rather, Dr. Van Ness opines that “Kaper teaches applications of its technique to cancer” and from this leaps to the conclusion that “it would have been natural and obvious to apply the method to cell-free DNA.”¹⁷⁵ I disagree. It is true that cfDNA, including tumor-associated cfDNA, was known to exist.¹⁷⁶ However, the use of tissue

¹⁷³ See Van Ness Decl. at ¶ 237.

¹⁷⁴ Additionally, Dr. Van Ness mischaracterizes my deposition testimony, which he attempts to use to interpret the '035 Patent claims. Dr. Van Ness stated that, “[a]ccording to [me], the first element of the claim, 1[a] is limited only to the first cycle of amplification.” Van Ness Decl. at ¶ 250. This statement is incorrect as I did not testify that claim 1[a] of the '035 Patent is limited to the first PCR cycle only. In fact, my testimony was “[w]ell, I think as I’ve testified, it does happen in the first cycle; but it can also happen in subsequent cycles.” See Dkt. 116-6 at 103:17-104:24.

¹⁷⁵ See Van Ness Decl. at ¶ 240.

¹⁷⁶ *Id.* at ¶¶ 241-247. Dr. Van Ness cites certain references for the proposition that “use of cell-free DNA in cancer monitoring and detection was well-known,” without explaining how it would have been obvious to modify Kaper’s disclosure using such references. See

sources of tumor-associated DNA would not render obvious the use of cfDNA in the claimed method because, for example, as explained in greater detail *supra*,¹⁷⁷ tumor tissue samples contain tumor-associated DNA at a high abundance in contrast to cfDNA.

158. The Van Ness Declaration fails to support a contrary conclusion for several reasons. First, as Dr. Van Ness admitted in deposition, Kaper (2010) Poster itself does not “describe how to modify multiplex amplification in order to address problems that are particular to cell-free DNA as opposed to genomic DNA.”¹⁷⁸ Nor does the Van Ness Declaration identify any specific teaching in other prior art references that could be used to modify the Kaper (2010) Poster’s approach for non-cfDNA successfully with respect to tumor-associated SNPs, or why a skilled artisan would have been motivated to do so. Second, the Van Ness Declaration does not explain why one of ordinary skill in the art would have reasonably expected success in arriving at the claimed combination in light of the technical challenges associated with manipulating cfDNA.

159. These known difficulties in manipulating cfDNA are consistent with the limitations of the Fluidigm Access Array that are specifically reported in the ’035 Patent (quoted *infra*), and which would have been before the Examiner during prosecution of the ’035 Patent application. Despite these obstacles to success specifically described in the

id. at ¶¶ 241 (Cantor), ¶ 242 (Ehrich), ¶ 243 (Lo). In deposition, for example, Dr. Van Ness could not recall whether those references taught multiplex amplification. Van Ness Tr. 151:8-152:23.

¹⁷⁷ See Section II.

¹⁷⁸ See Van Ness Tr. at 149:6-22.

'035 Patent, the Van Ness Declaration does not address the '035 Patent's disclosure or explain how one of ordinary skill in the art would have reasonably expected to succeed in using the Kaper (2010) Poster's specific approach to amplify tumor-associated cfDNA (ctDNA) or what modifications would have been necessary to achieve that particular goal in light of the unpredictability and technical challenges in working with ctDNA.

160. Specifically, the '035 Patent explicitly distinguishes its invention from the use of the Fluidigm Access Array equipment that is described in the Kaper (2010) Poster. The inventors explain that the Access Array approach is *not* desirable for amplifying samples that only contain "a limited amount of DNA." As explained *supra*,¹⁷⁹ tumor-associated cfDNA is relatively low-abundance among the other cfDNA (from healthy cells) in a person's plasma sample, and the levels of ctDNA in a plasma sample are also variable. As one of ordinary skill in the art would have recognized, the results from using such low-abundance samples will be biased if the Access Array's uniplex reactions does not contain the target DNA that is the subject of the amplification. This problem is compounded when the approach is intended to amplify multiple loci that have different sequences ("polymorphic" loci), which can be present in tumor DNA sequences as those sequences are frequently mutated relative to healthy DNA. The '035 Patent recognizes these limitations with use of the Access Array, and the unpredictability in the art of using cfDNA and ctDNA as a source material for amplification.

¹⁷⁹ See Section II.

In general, to perform targeted sequencing of multiple (n) targets of a sample (greater than 50, greater than 100, greater than 500, or greater than 1,000), *one can split the sample into a number of parallel reactions that amplify one individual target. This has been performed in PCR multiwell plates or can be done in commercial platforms such as the FLUIDIGM ACCESS ARRAY* (48 reactions per sample in microfluidic chips) or DROPLET PCR by RAIN DANCE TECHNOLOGY (100 s to a few thousands of targets). Unfortunately, *these split-and-pool methods are problematic for samples with a limited amount of DNA*, as there is often not enough copies of the genome to ensure that there is one copy of each region of the genome in each well.

This is an especially severe problem when polymorphic loci are targeted, and the relative proportions of the alleles at the polymorphic loci are needed, as the stochastic noise introduced by the splitting and pooling will cause very poorly accurate measurements of the proportions of the alleles that were present in the original sample of DNA.

Described here is a method to effectively and efficiently amplify many PCR reactions that is applicable to cases where only a limited amount of DNA is available. In an embodiment, the method may be applied for analysis of single cells, body fluids, mixtures of DNA such as the free floating DNA found in maternal plasma, biopsies, environmental and/or forensic samples.¹⁸⁰

In acknowledging these problems, the '035 Patent reports that its methods are comparatively advantageous when amplifying low-abundance DNA, including “free floating” cfDNA found in plasma.

161. The Van Ness Declaration does not address the '035 Patent's description of its methods' advantages over the Access Array, as the use of that equipment is described

¹⁸⁰ See '035 Patent at 85:21-46 (emphases added).

in the Kaper (2010) Poster. Nor does Dr. Van Ness identify with any specificity how one of ordinary skill in the art would modify the Kaper (2010) Poster's approach to achieve the claimed inventions.

162. The Van Ness Declaration also does not explain how one of ordinary skill in the art would have reasonably expected to succeed in doing so. As explained *supra*, these modifications would have been challenging to one of ordinary skill in the art at least because manipulating tumor-associated cfDNA would have been relatively difficult due, for example, to its fragmented nature and low abundance in plasma samples. Instead of explaining how one of ordinary skill in the art would have overcome these challenges and why one of ordinary skill in the art would have been motivated to do so, Dr. Van Ness combines disparate, unrelated teachings in the prior art without explaining why one of ordinary skill in the art would have been motivated to combine those particular teachings as opposed to the myriad other disclosures available to one of ordinary skill in the art. Dr. Van Ness's approach is therefore more consistent with the impermissible use of hindsight rather than what would have been obvious to a skilled artisan considering the Kaper (2010) Poster without the benefit of the '035 Patent's disclosure.

163. There are additional reasons why the use of cancer gene exons in the Kaper (2010) Poster does not render obvious the use of cell-free DNA as well. Setting aside the use of the problematic Fluidigm approach, the PCR data in Figure 3 of the Kaper (2010) Poster further teaches away from the use of cell-free DNA. The Kaper (2010) Poster's PCR product sizes, based on the figure's size markers, indicate that 46 of the 48 (96%) are

equal to or greater than 200 base pairs and that 16 of the 48 (33%) are equal to or greater than 300 bp. In contrast, it was known the cell-free DNA has a typical size range of 140-170 base pairs.¹⁸¹ Simply put, the PCR product sizes, and therefore the distance of the primer pair sets used in the Kaper (2010) Poster, are too large for use with cfDNA and would have required a complete overhaul and redesign of the Kaper (2010) Poster approach to manipulate cfDNA—something not taught in any reference cited by Dr. Van Ness.

(b) Claim 1[b]-[c] of the '035 Patent

amplifying the tagged products one or more times to generate final amplification products, wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume, wherein one of the amplifying steps introduces a barcode and one or more sequencing tags; and

sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products, wherein the plurality of SNP loci comprises 25-2,000 loci associated with cancer.

164. The Van Ness Declaration contends that the Kaper (2010) Poster renders obvious the claim 1[b] limitation.¹⁸² I disagree. To begin with, the Kaper (2010) Poster provides no disclosure whatsoever of targeted amplification of SNPs, much less SNPs associated with cfDNA.

¹⁸¹ See, e.g., Forshew (2012) at 1 (“Circulating DNA is fragmented to an average length of 140 to 170 base pairs (bp).”).

¹⁸² See Van Ness Decl. at ¶ 249.

165. Indeed, the Van Ness Declaration acknowledges that the Kaper (2010) Poster does not disclose the limitation “wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume.”¹⁸³ I agree with this assessment: the Kaper (2010) Poster does not teach amplifying a plurality of SNP loci in a single reaction volume, particularly using cfDNA containing such SNPs which would have been challenging as explained *supra*.

166. The primers used in the Kaper (2010) Poster are used to identify the presence of certain genes in tumor tissue samples (“cancer gene exons”). It might be theoretically feasible to use the Kaper (2010) Poster’s Access Array to amplify DNA sequences containing SNP loci instead, but this is not taught or suggested and doing so would require substantial effort to redesign the Kaper (2010) Poster’s approach. For example, to identify SNPs, one of ordinary skill in the art would have needed to redesign the Kaper (2010) Poster’s primers to target SNP loci. The Van Ness Declaration does not explain why one of ordinary skill in the art, even if they had such an idea, would have reasonably expected to succeed in pursuing any particular approach to modifying the Fluidigm Access Array’s design and methods to achieve this goal.

167. The Van Ness Declaration’s citations to general teachings in the art or by other witnesses fail to establish a different conclusion.¹⁸⁴ For instance, those citations do

¹⁸³ *Id.* at ¶ 252.

¹⁸⁴ *Id.*, *e.g.*, at ¶¶ 253-256. Dr. Van Ness argues that “SNPs were well-known in the art” (*id.* at ¶¶ 253-254), also citing to Ehrich & Van der Boom, *Restriction endonuclease*

not address the unpredictability and known technical challenges in the art associated with manipulating cfDNA, much less teach anything specific about (or even related to) modifying the Kaper (2010) Poster approach. Therefore, these sources do not support a finding that one of ordinary skill in the art would have reasonably expected success in modifying the Kaper (2010) Poster to achieve the methods recited in the '035 Patent claims.

168. For essentially the same reasons regarding claim 1[b], I disagree with Dr. Van Ness's opinions that the Kaper (2010) Poster renders obvious claim 1[c] of the '035 Patent,¹⁸⁵ which recites sequencing SNPs, including 25-2,000 SNPs as required by the '035 Patent claims.¹⁸⁶ Again, Dr. Van Ness fails to show that the Kaper (2010) Poster teaches this limitation (as it does not) and instead suggests that it would have been obvious to do so. I disagree. The Kaper (2010) Poster's disclosure of sequencing 480 cancer gene exons,

enhanced polymorphic sequence detection, PCT International Patent Publication No. WO 2008/118988 (2008) (the "PCT '988 Appl. (Ehrich)") (*id.* at ¶ 254), Cantor, *Method for non-invasive prenatal diagnosis*, PCT International Patent Publication No. WO 2005/023091 (2005) (the "PCT '091 Appl. (Cantor)") (*id.* at ¶ 255), and Dr. Quackenbush's testimony in the *CareDx* trial (*id.* at ¶ 256).

¹⁸⁵ See Van Ness Decl. at ¶ 260.

¹⁸⁶ I understand that although the Court has not construed the '035 Patent claims, the claims recite that "one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume" and, in a subsequent step, that "the plurality of SNP loci comprises 25-2,000 loci associated with cancer." To the extent that "the plurality of SNP loci" refers to "a plurality of single nucleotide polymorphism (SNP) loci" recited in the amplification step, Kaper (2010) Poster does not teach or suggest this method. Kaper (2010) Poster teaches only multiplex amplification of 10 targets (not SNPs) in a single reaction volume in a preamplification step. Performing a greater number of multiplex amplification reactions in a single volume would therefore require redesigning the Kaper (2010) Poster approach.

absent more, would not render obvious an approach of sequencing 25-2,000 SNP loci in particular, especially SNP loci present on cfDNA. Indeed, other well-known gene alterations exist in cancers including, but not limited to, structural variants and copy-number variants.

169. Relatedly, the Van Ness Declaration contends that “higher degrees of multiplex were commonplace in the art.”¹⁸⁷ Even assuming this were true, Dr. Van Ness does not explain why it would have been obvious to modify the Kaper (2010) Poster approach using the (problematic) Fluidigm Access Array in order to achieve increased multiplexing capability, much less for the purpose of performing targeted amplification of a plurality of SNP loci in a single reaction volume. I disagree that any of these references support Dr. Van Ness’s obviousness opinions.

(ii) *The Combination of ’183 Patent Pub. (Han) and Wang (2010) (“ARM-PCR”) Does Not Render the ’035 Patent Claims Obvious*

170. At least for the reasons explained *infra*, Dr. Van Ness has failed to show that the ’035 Patent claims are obvious over the teachings of the combination of the ’183 Patent

¹⁸⁷ See Van Ness Decl. at ¶ 263. In support of this opinion, Dr. Van Ness also cites to Varley & Mitra, *Nested patch PCR enables highly multiplexed mutation discovery in candidate genes*, GENOME RESEARCH 18:1844-1850 (2008) (“Varley (2008)”) at ¶ 264; cites to ’038 Patent Pub. (Mir) (*i.e.*, Fluidigm system) at ¶ 265; cites to Broude, *Novel compositions and methods for carrying out multiplex PCR reactions on a single sample*, U.S. Patent Application Publication No. US 2004/0126760 (2004) (the “’760 Patent Pub. (Broude)”) at ¶ 266; cites to Lyamichev *et al.*, *Amplification methods and compositions*, U.S. Patent No. 7,790,393 (2010) (the “’393 Patent (“Lyamichev”)” at ¶ 267; and cites to Shoemaker *et al.*, *Rare cell analysis using sample splitting and DNA tags*, PCT International Patent Publication No. WO 2007/147079 (2007) (the “PCT ’079 Appl. (“Shoemaker”)” at ¶ 268. Dr. Van Ness noted in deposition that Varley (2008) did not use cfDNA. Van Ness Tr. at 138:137:20-138:9.

Pub. (Han)¹⁸⁸ and Wang (2010).¹⁸⁹ I disagree with Dr. Van Ness's opinions regarding their combination.

171. Dr Van Ness's attempt to combine the teachings of '183 Patent Pub. (Han) and Wang (2010) is flawed in multiple respects. Neither reference teaches or suggests all limitations of the '035 Patent's claimed methods, which Dr. Van Ness does not dispute. This alone renders the combination insufficient.

172. Nevertheless Dr. Van Ness purports to combine these references in an attempt to use the teachings of one to remediate the gaps in the other's disclosure. As explained *infra*, Dr. Van Ness's analysis fails because the teachings of the two references do not complement each other in such a way that one of ordinary skill in the art would have either been motivated to achieve the claimed invention or would have reasonably expected to succeed in doing so, including given the unpredictability and known technical challenges in the art in manipulating ctDNA. I reserve the right to offer additional opinions regarding the '035 Patent in view of these references, including in combination with any other art.

¹⁸⁸ See Dkt. 116-16, Han, *Amplicon rescue multiplex polymerase chain reaction for amplification of multiple targets*, U.S. Patent Publication No. US 2009/0253183 (2009) (the "'183 Patent Pub. (Han)").

¹⁸⁹ See Dkt. 117-4, Wang *et al.*, *High throughput sequencing reveals a complex pattern of dynamic interrelationships among human T cell subsets*, PROCEEDINGS OF THE NATIONAL ACADEMY OF SCIENCES OF THE UNITED STATES 107:1518-1523 (2010) ("Wang (2010)").

(a) Claim 1[a] of the '035 Patent

tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products, wherein the isolated cell-free DNA is isolated from a blood sample collected from a subject who is not a pregnant women

173. I disagree with Dr. Van Ness's opinion that the combination of '183 Patent Pub. (Han) and Wang (2010) render obvious Claim 1[a] of the '035 Patent.¹⁹⁰ As a preliminary matter, '183 Patent Pub. (Han) does not describe the performance of its methods on cfDNA. Rather, '183 Patent Pub. (Han) teaches that ARM-PCR can be useful to amplify DNA from certain specific cellular and viral sources that do not include cfDNA or even DNA from plasma samples.

The inventor has developed a new method for amplifying nucleic acids that may be used to detect the presence, and relative amounts present, of *nucleic acids from viruses, bacteria, fungi, plant and/or animal cells* for the evaluation of medical, environmental, food, and other samples to identify microorganisms and other agents within those samples.¹⁹¹

174. Manipulating cfDNA—and particularly low abundance and highly variable ctDNA, an unpredictable source material for amplification—presented significant technical difficulties, as described *supra*.¹⁹² The '183 Patent Pub. (Han) does not teach or explain how its methods can be used successfully for any purpose with respect to cfDNA, and Dr. Van Ness does not contend otherwise.

¹⁹⁰ See Van Ness Decl. at ¶ 278.

¹⁹¹ See '183 Patent Pub. (Han) at [0009] (emphasis added).

¹⁹² See Section II.

175. The Van Ness Declaration also does not explain why one of ordinary skill in the art would have been motivated to use cfDNA in '183 Patent Pub. (Han)'s methods, assuming it would even be feasible to do so. Rather, to overcome '183 Patent Pub. (Han)'s lack of disclosure of cfDNA, Dr. Van Ness points to phrases such as “clinical diagnosis” (mentioned once) and “detection of genetic disorder, and/or the detection of disease conditions” to render obvious the use of cfDNA.¹⁹³ But these generalized statements in '183 Patent Pub. (Han) do not refer to cfDNA, nor suggest its use. Wang (2010) likewise also does not teach or suggest using cfDNA and Dr. Van Ness does not contend otherwise.¹⁹⁴

176. The Van Ness Declaration attempts to overcome these undisputed deficiencies, arguing once more that that “the use of cell-free DNA in cancer monitoring and detection was well-known.”¹⁹⁵ These general citations do not rescue the Van Ness Declaration's faulty opinions regarding the '035 Patent claims' obviousness because Dr. Van Ness does not explain—for any cited reference—why one of ordinary skill in the art would have been motivated to use cfDNA in '183 Patent Pub. (Han)'s method, how Han's method would need to be modified to do so, or why one of ordinary skill in the art would have reasonably expected success in making the claimed combination given the known

¹⁹³ See Van Ness Decl. at ¶ 280.

¹⁹⁴ *Id.* at ¶¶ 278-280.

¹⁹⁵ *Id.* at ¶ 280, citing to ¶¶ 241-248 in the Van Ness Decl. that refer to Cantor, (¶ 241), Ehrich (¶ 242), Lo (¶ 243), myself (¶ 244), Natera (¶ 245), and Quackenbush (¶¶ 246-247)).

technical challenges in working with tumor-associated cfDNA and unpredictability of results of such approaches, as discussed *supra*.

(b) Claim 1[b] of the '035 Patent

amplifying the tagged products one or more times to generate final amplification products, wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume, wherein one of the amplifying steps introduces a barcode and one or more sequencing tags

177. I disagree with Dr. Van Ness's assertion that "Han teaches amplifying the products one or more times wherein one of the steps comprises targeted amplification of SNP loci in a single reaction volume."¹⁹⁶ For one thing, '183 Patent Pub. (Han) does not mention the terms "single nucleotide polymorphism" or SNP even once.¹⁹⁷

178. Moreover, in my opinion one of ordinary skill in the art would not have found it obvious to modify '183 Patent Pub. (Han)'s approach to amplify or identify SNPs. In addition to the well-known challenges in manipulating SNP-containing cfDNA explained *supra*, '183 Patent Pub. (Han) teaches the use of ARM-PCR for the purpose of improving molecular differential diagnostic (MDD) assays.¹⁹⁸ As taught in '183 Patent Pub. (Han), this approach represents a binary assay that reads out either the presence or absence of

¹⁹⁶ *Id.* at ¶¶ 282-283.

¹⁹⁷ *See* '183 Patent Pub. (Han) throughout.

¹⁹⁸ *Id.* at [0003]-[0004].

targeted sample types (*e.g.*, the cells and viruses listed *supra*, not SNPs). The examples provided in '183 Patent Pub. (Han) illustrate the results of the ARM-PCR assay.¹⁹⁹

179. In contrast, targeting SNPs does not involve a binary assay. For example, there may be any of four base pairs at a given SNP locus. The Van Ness Declaration does not explain how the '183 Patent Pub. (Han)'s binary method (merely detecting presence versus absence) could be used with respect to targeting SNPs (which have specific sequences). Dr. Van Ness also does not explain how one of ordinary skill in the art would have reasonably expected success in modifying it for this purpose, and, accordingly, the Van Ness Declaration has also failed to demonstrate that '183 Patent Pub. (Han) discloses this limitation.

180. Although Dr. Van Ness asserts that “Wang also teaches this limitation,”²⁰⁰ I disagree. Like '183 Patent Pub. (Han), Wang (2010) does not once mention the terms “single nucleotide polymorphism” or SNP,²⁰¹ much less in the combination of the claimed methods. Rather, Wang (2010) teaches the use of ARM-PCR as a semiquantitative PCR amplification method to maintain the representation of CDR3 sequences obtained from different subsets of T-cells.²⁰² One of ordinary skill in the art would have understood that Wang (2010) was attempting to amplify the same region (the CDR3 sequence) in each of

¹⁹⁹ *Id.* at [0026], [0029]-[0052].

²⁰⁰ *See* Van Ness Decl. at ¶ 284.

²⁰¹ *See* Wang (2010) throughout.

²⁰² *Id.* at 1518, 1520.

the T-cell population subsets. The consequence of Wang (2010)’s approach is that one of ordinary skill in the art would have understood Wang (2010) to teach only using a *single pair of PCR primers*—*e.g.*, a forward primer and a reverse primer that can be used to amplify the same CDR3 region on the different subsets of T-cells—*not* a “targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume” as claimed. The latter approach would require *different* primer pairs to amplify *different* SNP loci (*i.e.*, a plurality of these loci), which Wang does not teach or suggest (and in fact teaches away from).

181. Thus, Dr. Van Ness has not demonstrated that Wang (2010)’s disclosure suggests, much less discloses, this limitation. Dr. Van Ness’s citation to the knowledge of one of ordinary skill in the art to further remediate this deficiency²⁰³ further fails at least for the same reasons explained *supra*—*e.g.*, Dr. Van Ness does not explain how one of ordinary skill in the art would have reasonably expected success doing so given the known technical challenges in working with tumor-associated cfDNA.

²⁰³ See Van Ness Decl. at ¶ 285.

(c) **Claim 1[c] of the '035 Patent**

sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products, wherein the plurality of SNP loci comprises 25-2,000 loci associated with cancer

182. Dr. Van Ness opines that this limitation is obvious as Wang (2010) teaches “sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products.”²⁰⁴ I disagree.

183. As a preliminary matter, '183 Patent Pub. (Han) does not disclose sequencing, and Dr. Van Ness does not contend otherwise. The Van Ness Declaration does not acknowledge this deficiency besides combining '183 Patent Pub. (Han) with Wang (2010), which does disclose sequencing. However, one of ordinary skill in the art would not have been motivated to modify '183 Patent Pub. (Han)'s approach with Wang (2010)'s methods to achieve the claimed invention, and would not have reasonably expected to succeed in doing so, for the reasons explained *supra* with respect to Claim 1[b]—namely, Wang (2010) teaches amplification of *one* locus (CDR3) in different cell populations and does not suggest, much less disclose, “targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume.”

184. Dr. Van Ness points to the '183 Patent Pub. (Han) for the proposition that the '183 Patent Pub. (Han) teaches “that the plurality of SNP loci comprises 25-2,000 loci associated with cancer”—specifically, that Table 2 of Han demonstrates that this technique

²⁰⁴ *Id.* at ¶¶ 295-296.

is capable of the degree of multiplex recited in the claim.”²⁰⁵ According to Dr. Van Ness, this “Table displays 72 forward and reverse primers which when run in a single volume reaction would be within the multiplex range recited in this claim.” I disagree as Dr. Van Ness is misreading the teachings of the ’183 Patent Pub. (Han). Specifically, Figure 1 of ’183 Patent Pub. (Han) shows that each locus requires six primers—three forward primers, and three reverse primers. Applying this teaching, ’183 Patent Pub. (Han)’s approach teaches a maximum of targeting 12 loci, not 72 as Dr. Van Ness contends (six primers per loci x 12 loci = 72 total primers). And neither ’183 Patent Pub. (Han) nor Wang (2010) teaches or suggests targeting any higher number of loci.

VII. THE NATERA PATENTS ARE VALID UNDER SECTION 101

185. Dr. Van Ness opines that the claims of the ’454 and ’035 Patents are all directed to patent-ineligible subject matter.²⁰⁶ I disagree.

186. As a preliminary matter, Dr. Van Ness’s opinions are conclusory and based on a misreading of the claims—indeed, Dr. Van Ness’s opinions ignore the claims’ plain language. The claims on their face say they are directed to methods of preparing non-natural preparations, which I understand is patentable subject matter under controlling Federal Circuit precedent. The Van Ness Declaration’s analysis is also flawed in that it is based on limited sets of information. Dr. Van Ness relies on out of context positions taken by Natera in other proceedings, regarding irrelevant CareDx/Stanford patents that are not

²⁰⁵ *Id.* at ¶ 298.

²⁰⁶ *See* Van Ness Decl. at § XI.

related to the patents at issue, with claims that are very different, and cherry-picked statements in the patents themselves.²⁰⁷ I disagree with Dr. Van Ness's approach and his conclusions. For example, statements Natera purportedly made about individual techniques made in other proceedings are not relevant at least to the extent that, as I understand from counsel, the character of the claims as a whole—*i.e.*, the use of techniques in the specific context recited in the claims—must be considered in determining eligibility.

187. As discussed *infra*, the Asserted Claims of the '454 and '035 Patents represent new method of preparation methods for achieving useful results. Similarly, these claimed methods are not routine, conventional, or well-understood, at least when they are considered in the recited ordered combinations.²⁰⁸ The Van Ness Declaration fails to evaluate the claimed methods according to the appropriate standards, which I have been informed and understand represent the proper framework for the eligibility analysis.

188. I provide my specific opinions *infra* regarding patent-eligibility of the Asserted Claims of the Natera Patents at step one and (if the Court reaches step two) at step two of the patent-eligibility analysis. I reserve the right to offer additional opinions at another time.

²⁰⁷ *Id.* throughout.

²⁰⁸ As noted *supra*, Dr. Van Ness has not located any prior art reference that even arguably discloses all of the claimed limitation of any claim, and thus is not alleging any claim is anticipated under Section 102 of the Patent Act.

A. The '454 Patent Claims Are Patent-Eligible

(i) *The '454 Patent Claims Are Directed to Patent-Eligible Subject Matter*

189. Dr. Van Ness opines that the '454 Patent claims are “methods of detection” claims.²⁰⁹ I disagree. As a preliminary matter, I am unaware that patent claims for methods of preparation useful for detecting SNVs are necessarily patent-ineligible and the Van Ness Declaration does not identify such a principle.

190. Regardless, from my understanding of principles governing the “directed to” analysis, I believe Dr. Van Ness’s analysis is flawed. It appears that Dr. Van Ness reaches these opinions by focusing on certain claim limitations and excluding others. I am not aware of a basis to exclude claim limitations in determining what claims are directed to. Rather, I understand from counsel that the “directed to” analysis requires consideration of the character of the claims as a whole. I note that the Van Ness Declaration does not assert that Dr. Van Ness is considering the character of the claims as a whole. Additionally, I understand from Dr. Van Ness’s testimony that he believes the Natera Patent claims are ineligible because they “involve[]” detecting a natural phenomenon, such as DNA.

Q. And in determining, for your opinions in this case, whether the Natera patents claims are directed to a natural phenomenon, did you simply look as to whether the claims involved natural phenomenon? Is that how you did it?

A. That was one of the ways that I looked to see to see if it was directed to a natural product. The claims define the scope of the invention, and *the scope of the invention*

²⁰⁹ See Van Ness Decl. at ¶¶ 305-310.

is related to whether that is involved in detecting a natural product. So, yes, I did look at the claims to help inform that decision.²¹⁰

I am informed by counsel that the patent-eligibility determination requires more than determining if the claims “involve” an ineligible concept. Rather, claims may be eligible for patenting if they recite a method for achieving a new and useful result.

191. In my opinion, the Asserted Claims of the '454 Patent are directed not to a natural phenomenon, but rather to an inventive combination of specific laboratory techniques, each of which requires significant human effort to perform. The combination of techniques recited in the Asserted Claims of the '454 Patent represents a novel and innovative improvement over earlier methods of sample preparation, and which achieves superior results. I discuss the advantages of the claimed methods in greater detail *infra* with respect to step two of the eligibility analysis.

192. Notably, when performed in combination as recited in Asserted Claims of the '454 Patent, the claimed techniques recite methods for preparing both tumor DNA samples and cfDNA samples in specific ways. This claimed method therefore represents a new and useful method of sample preparation that can be used to yield a tangible and useful result. This result is only achieved by the combination of specific laboratory techniques that, together, represent a new and useful sample preparation method. The Van Ness Declaration does not address the new and useful result achieved by the claimed

²¹⁰ See Van Ness Tr. at 59:10-23 (emphasis added).

combination of steps. Nor does this section of the Van Ness Declaration discuss certain specific limitations recited in the claims, which I consider *infra*.

193. First, the Van Ness Declaration does not address that, in the '454 Patent preamble, the Asserted Claims expressly recite that they involve “[a] method of preparing a sample.”²¹¹ I believe this limitation should be considered at least because, indeed, the Asserted Claims of the '454 Patent recite a specific protocol for preparing a sample and that is what they are directed to. I discuss its steps in turn *infra*. One of the method’s steps is a detecting step, but that is not the only step. Indeed, detection is only possible because of the performance of the other steps of the claimed method that precede detection. Dr. Van Ness acknowledged at his deposition that the '454 Patent claims recite methods of DNA manipulation. Dr. Van Ness also admitted that the '454 Patent claims recite amplifying specific numbers of SNV loci.

Q. Is it your contention that – I’m not asking you whether you think they’re routine and conventional, just yes or no whether they’re there. Is it your contention that Claim 1 of the '454 patent does not recite any methods for manipulating DNA, amplifying and sequencing?

A. Claim 1 does discuss manipulating and performing procedures on DNA and amplifying.²¹²

Q. Okay. Do you disagree that the claims recite a specific number of loci that are amplified?

A. ***It provides a range of 10 to 500.***²¹³

²¹¹ See Van Ness Decl. at ¶¶ 314-317.

²¹² See Van Ness Tr. at 88:25-89:11 (objection and colloquy omitted).

²¹³ *Id.* at 91:10-13 (emphasis added).

194. Second, the first-recited step of the Asserted Claims of the '454 Patent requires performing one of two specific sequencing techniques—whole genome sequencing or whole exome sequencing—on a subject's tumor sample in order to identify a plurality of tumor-specific SNV mutations. Both techniques require significant human effort and intervention to achieve. For example, I reproduce information *infra* about these methods from a 2010 review²¹⁴ I published regarding the use of next-generation sequencing platforms to accomplish these techniques—a significant technical endeavor. One of ordinary skill in the art would have understood that performance of these specific sequencing techniques achieves a new and useful result—namely, the identification of a cancer patient's genomic sequences, either throughout the whole genome or in the entire coding regions of the genome (*i.e.*, all of the exons). Dr. Van Ness agrees, as he testified at his deposition.

Q. Thank you. And do you – strike that. You would agree that whole genome sequencing or whole exome sequencing of DNA requires human intervention and effort to perform?

A. DNA doesn't sequence itself. ***It does take somebody to do the process of the sequencing.***²¹⁵

195. As the Asserted Claims of the '454 Patent recite, these comprehensive sequencing approaches (compared to more limited approaches, *e.g.*, identifying a few

²¹⁴ See Ex. 8, Metzker, *Sequencing technologies — the next generation*, NATURE REVIEWS GENETICS, 11: 31-46 (2010) (“Metzker 2010”).

²¹⁵ See Van Ness Tr. at 89:14-21 (objection omitted, emphasis added).

cancer-associated biomarkers) permit identification of multiple tumor-associated SNVs which will be unique for each subject. Thus, the claims do not involve merely “sequencing,” as Dr. Van Ness suggests.²¹⁶ “Sequencing” alone would not have been adequate to provide new and useful comprehensive information about a subject’s genome. Rather, the ’454 Patent’s method—all of the recited steps—must be performed using the specific recited techniques in order to obtain the genomic information needed to achieve a new and useful result.

196. As referenced *supra*, my 2010 review exemplifies how the use of next-generation sequencing (also known as massively parallel sequencing) platforms to accomplish whole genome sequencing and whole exome sequencing involved significant effort to design and implement—far more than merely “sequencing.”

The inexpensive production of large volumes of sequence data is the primary advantage over conventional methods. Here, I present a technical review of template preparation, sequencing and imaging, genome alignment and assembly approaches, and recent advances in current and near-term commercially available NGS instruments.²¹⁷

The ability to sequence the whole genome of many related organisms has allowed large-scale comparative and evolutionary studies to be performed that were unimaginable just a few years ago.²¹⁸

Here, I present a technical review of template preparation, sequencing and imaging, genome alignment and assembly, and current NGS platform performance to provide guidance on

²¹⁶ *Id.*, e.g., at ¶¶ 318-328.

²¹⁷ See Metzker 2010 at Abstract.

²¹⁸ *Id.* at 31.

how these technologies work and how they may be applied to important biological questions. I highlight the applications of human genome resequencing using targeted and whole-genome approaches, and discuss the progress and limitations of these methods, as well as upcoming advances and the impact they are expected to have over the next few years.²¹⁹

Despite the substantial cost reductions associated with NGS technologies in comparison with the automated Sanger method, whole-genome sequencing is still an expensive endeavour (see below). An interim solution to this problem may be to use NGS platforms to target specific regions of interest. This strategy can be used to examine all of the exons in the genome, specific gene families that constitute known drug targets or megabase-size regions that are implicated in disease or pharmacogenetics effects through genome-wide association studies. Although any of the NGS platforms can perform the sequencing phase of targeted capture, some may be better suited than others (Table 1).²²⁰

197. In addition to misapprehending the nature of the Asserted Claims, the Van Ness Declaration's analysis is deficient in that Dr. Van Ness does not address whole genome sequencing or whole exome sequencing in his analysis of what the Asserted Claims of the '454 Patent are directed to. Nor does Dr. Van Ness explain his reasoning for ignoring these claim limitations and others.

198. Third, beyond sequencing, the Asserted Claims of the '454 Patent additionally recite a specific protocol for amplifying cfDNA.²²¹ This step requires one of ordinary skill in the art to perform at least five specific activities, each of which requires

²¹⁹ *Id.*

²²⁰ *Id.* at 41 (internal citations omitted).

²²¹ *See* '454 Patent at 171:32-38.

significant human effort to design and implement. Specifically, the Asserted Claims of the '454 Patent require [1] performing targeted multiplex amplification [2] which has been designed to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA [3] isolated from a plasma sample of the subject or DNA derived therefrom [4] to obtain amplicons having a length of 50-150 bases, [5] wherein the target loci are amplified together in the same reaction volume.²²²

199. Notably, targeted amplification in one reaction volume is one way to amplify DNA (using human effort), but not the only way, as Dr. Van Ness acknowledged in his deposition.

Q. Thank you. You would agree that targeted multiplex amplification to amplify 10 to 500 target loci, each encompassing a different tumor-specific SNV mutation, requires human intervention and effort to perform, correct?

A. Yes, there are a lot of humans that can do that, sure.

Q. Okay. Do you disagree that claim 1 of the '454 patent recites a specific kind of amplification, which is targeted multiplex amplification?

A. It – it states that “Wherein” – “the target loci are amplified together in the same reaction.” So there is a statement there of amplifying target loci.²²³

Q. Right. But targeted amplification is not the only way to amplify DNA, right? Are there other ways to do it?

²²² *Id.*

²²³ *See* Van Ness Tr. at 89:22-90:6 (objection and clarification by court reporter omitted).

A. *Sure*.²²⁴

Q. And there are ways of – of amplifying multiple targets without amplifying them in the same reaction volume, right?

A. You can amplify a target, one in every individual tube. *Certainly there are ways to amplify a single target in one tube and a different target in another tube*.²²⁵

200. In deposition, as previously discussed (Section II, *supra*), Dr. Van Ness acknowledged particular and “unique” challenges associated with multiplex amplification of cfDNA, in discussing amplification of different types of cfDNA (*e.g.*, cfDNA from patients who received an organ transplant, or pregnant women, or subjects with cancer).²²⁶ Dr. Van Ness admitted, for example, that “it’s not sufficient simply to isolate cell-free DNA” in order to perform multiplex amplification in light of the challenges of working with cfDNA.²²⁷ As Dr. Van Ness acknowledged, depending on the context of working with particular cfDNA, different protocols “might be required for successful multiplex amplification.”²²⁸

201. In combination, these approaches achieve a new and useful result of efficiently amplifying a subject’s tumor-associated SNVs together. Notably, the targeted amplification process generates DNA that did not previously exist in nature; that is, the

²²⁴ *Id.* at 91:3-9 (objection omitted, emphasis added).

²²⁵ *Id.* at 92:16-23 (objection omitted, emphasis added).

²²⁶ *Id.* at 64:4-66:6 (objections, court reporter clarification, and colloquy omitted).

²²⁷ *Id.* at 65:14-15.

²²⁸ *Id.* at 65:4-6.

amplicons which are modified with additional DNA sequences necessary for the following sequencing step. As explained *infra*, the specific claimed approach is also a material improvement over prior art techniques because it avoids dividing the plasma sample into individual reactions, an approach which would require more cfDNA starting material, and may lead to biased results when the sample contains very low, and/or variable levels of DNA, as in the case of ctDNA.

202. Fourth, the Asserted Claims of the '454 Patent recite ultra-deep sequencing—*i.e.*, sequencing to a depth of read of 50,000 or more for each target SNV locus.²²⁹ As explained *supra*, it was not common to sequence to this depth of read for a variety of reasons. Doing so was expensive and time-consuming and, although increasing read depth could under some conditions could improve sensitivity, ultra-deep sequencing would not necessarily be useful for any particular purpose. I discuss certain limitations of ultra-deep sequencing *supra* with respect to Forshew (2012). Thus, the combination of sequencing to the claimed depth of read with the other steps of the method represented a new and useful technique for sample preparation.

203. Accordingly, from my review of the Asserted Claims of the '454 Patent, in my opinion, their character as a whole is directed to eligible subject matter—a new and useful method for sample preparation using a combination of specific laboratory techniques. They are not, as Dr. Van Ness opines, directed to natural phenomenon or other

²²⁹ *Id.* at 171:39-43.

ineligible subject matter.²³⁰ Moreover, as explained *supra*, the “detecting” step is performed on DNA that did not exist in nature—*i.e.*, amplicons from isolated cfDNA.

204. In reaching his contrary conclusions, Dr. Van Ness discusses other patents from separate proceedings.²³¹ I disagree with his assessment of these non-asserted patents at least because those patents recite different limitations. The Van Ness Declaration does not offer any reasoned basis to equate their recited combination to the combination recited in the Asserted Claims of the ’454 Patent.

205. For all the aforementioned reasons, in my opinion that the Asserted Claims of the ’454 Patent, when considered as a whole, are directed to patent-eligible subject matter and step two of the patent-eligibility analysis is unnecessary. Even under an analysis of step two, the Asserted Claims of the ’454 Patent should still be found patent-eligible, as explained *infra*.

(ii) *The ’454 Patent Claims, Considered Individually and In Combination, Are Not Routine or Conventional*

206. The Van Ness Declaration opines that certain aspects of the claimed methods of the ’454 Patent—sample preparation, amplification, and sequencing—were routine and conventional.²³² I agree that, considered independently and in isolation, these general techniques were known in the art as of 2014-2015. This is not to say, however, that these methods were routine or conventional, particularly in the context of working with tumor-

²³⁰ See Van Ness Decl. at § XI.

²³¹ *Id.* at ¶ 310.

²³² *Id.* at ¶¶ 312-338.

associated cfDNA. In fact, as explained *supra*,²³³ this approach was challenging in many respects even after the '454 Patent's priority date due in a large part to the difficulties in identifying rare genetic variants over the background "noise" (errors) of the sample preparation and sequencing techniques.

207. In any event, in my understanding, even if the recited limitations were routine, conventional, or well-understood, that would not be sufficient to show patent ineligibility, as the claim limitations must be considered both individually and as their ordered combination. The Van Ness Declaration fails to consider the latter—whether the '454 Patent limitations are well-understood, routine, or conventional in their ordered combinations. In my opinion, these claim limitations when considered in their ordered combination are not well-understood, routine, or conventional. Thus, the Asserted Claims of the '454 Patent should be deemed patent-eligible if the Court reaches step two of the eligibility analysis.

208. When considered as an ordered combination, the method recited in the Asserted Claims of the '454 Patent is not routine or conventional at least because the method's ordered steps recite improved, inventive processes for sample preparation. The ordered combination of steps improves upon prior art approaches in several respects, as described *infra*.

²³³ See Section II.

209. First, the claimed methods recited in the Asserted Claims of the '454 Patent require use of the specific laboratory techniques whole genome sequencing or whole exome sequencing. This step provides holistic information about a specific person's genetic background—and not merely “sequencing,” which does not itself provide this information.

210. Second, the methods can be used to prepare small amounts of cancer-associated cfDNA, which are challenging to manipulate.²³⁴ The '454 Patent specification explains the inventiveness of the claimed approach:

In another aspect, the present invention generally relates, at least in part, to improved methods of detecting single nucleotide variations (SNVs). These improved methods include improved analytical methods, improved bioassay methods, and improved methods that use a combination of improved analytical and bioassay methods. *The methods in certain illustrative embodiments are used to detect, diagnose, monitor, or stage cancer, for example in samples where the SNV is present at very low concentrations ... such as circulating free DNA samples.* That is, these methods in certain illustrative embodiments are particularly well suited for samples where there is a relatively low percentage of a mutation or variant relative to the normal polymorphic alleles present for that genetic loci.²³⁵

In certain preferred embodiments the sample is a single cell sample or a plasma sample suspected of containing circulating tumor DNA. These embodiments take advantage of the discovery that by *interrogating DNA samples from single cells or plasma for CNVs and SNVs using the highly sensitive multiplex PCR methods disclosed herein, improved cancer detection can be achieved,* versus interrogating for

²³⁴ See Section II.

²³⁵ See '454 Patent at 44:6-21 (emphasis added).

either CNVs or SNVs alone ... The methods provided herein for detecting CNVs and/or SNVs in plasma of subjects ... provide the advantage of detecting CNVs and/or SNVs from tumors that often are composed of heterogeneous cancer cell populations in terms of genetic compositions. Thus, traditional methods, which focus on analyzing only certain regions of the tumors can often miss CNVs or SNVs that are present in cells in other regions of the tumor.²³⁶

211. Third, the specific claimed approach recited in the Asserted Claims of the '454 Patent is also an improvement over prior art techniques because amplifying the targeted loci together, when done successfully as the '454 Patent describes, is efficient. This approach has the additional benefit of avoiding dividing the plasma sample into individual reactions which may require more cfDNA starting material, lead to biased results when the sample contains very low, and/or variable levels of tumor-associated cfDNA.

Improved PCR amplification methods have also been developed that minimize or prevent interference due to the amplification of nearby or adjacent target loci in the same reaction volume (such as part of the sample multiplex PCR reaction that simultaneously amplifies all the target loci). These methods can be used to simultaneously amplify nearby or adjacent target loci, which is faster and cheaper than having to separate nearby target loci into different reaction volumes so that they can be amplified separately to avoid interference.²³⁷

The multiplex reaction methods provided herein, such as the massive multiplex PCR disclosed herein provide ***an exemplary process for carrying out the amplification reaction to help***

²³⁶ *Id.* at 147:22-45 (emphases added).

²³⁷ *Id.* at 103:2-11 (emphasis added).

*attain improved multiplexing and therefore, sensitivity levels.*²³⁸

In an embodiment, the particular primers that are most likely to cause unproductive side reactions may be removed from the primer library to give a primer library that will result in a greater proportion of amplified DNA that maps to the genome. *The step of removing problematic primers, that is, those primers that are particularly likely to form [sic] dimers has unexpectedly enabled extremely high PCR multiplexing levels* for subsequent analysis by sequencing.²³⁹

212. Fourth, the claimed methods recited in the Asserted Claims of the '454 Patent use the uncommon and expensive method of sequencing to a depth of read of 50,000 or more.

In certain aspects, the present invention provides a method for detecting cancer. The sample, it will be understood can be a tumor sample or a liquid sample, such as plasma, from an individual suspected of having cancer. *The methods are especially effective at detecting genetic mutations such as single nucleotide alterations such as SNVs, or copy number alterations, such as CNVs in samples with low levels of these genetic alterations as a fraction of the total DNA in a sample.* Thus the sensitivity for detecting DNA or RNA from a cancer in samples is exceptional. The methods can combine any or all of the improvements provided herein for detecting CNV and SNV to achieve this exceptional sensitivity.²⁴⁰

213. In addition to my opinions *supra*, I further note that the prior art cited by Dr. Van Ness under Section 103 does not show that any reference teaches each and every limitation of the claims, as arranged in the Asserted Claims of the '454 Patent. The fact

²³⁸ *Id.* at 64:58-62 (emphasis added).

²³⁹ *Id.* at 105:54-61 (emphasis added).

²⁴⁰ *Id.* at 63:53-65 (emphasis added).

that even combinations of references do not teach or suggest the claimed methods as recited in the Asserted Claims of the '454 Patent further supports my opinion that the ordered combination of the claims was not routine, conventional, or well-understood in the art.

B. The '035 Patent Is Patent-Eligible

(i) *The '035 Patent Claims Are Directed to Patent-Eligible Subject Matter*

214. Dr. Van Ness opines that the '035 Patent claims are “methods of detection” claims.²⁴¹ I disagree. “Detecting” is not even a recited step in the methods claimed in the '035 Patent.²⁴²

215. Regardless, from my understanding of principles governing the “directed to” analysis, I believe the Van Ness Decl.’s analysis is flawed in several respects. Dr. Van Ness reaches his opinion by focusing on certain claim limitations and excluding others. I am unaware of a basis to exclude claim limitations in determining what claims are directed to. Rather, I have been informed and I understand from counsel that the “directed to” analysis requires consideration of the character of the claims as a whole, which the Van Ness Decl. does not address. Indeed, Dr. Van Ness analyzes portions of the claims individually. Additionally, I understand from Dr. Van Ness’s testimony²⁴³ that Dr. Van Ness believes that the Asserted Claims of the '035 Patent are ineligible because they

²⁴¹ See Van Ness Decl. at ¶¶ 341-347.

²⁴² See, e.g., '035 Patent at 249:44-62.

²⁴³ See Van Ness Tr. at 59:10-23, shown *supra*.

involve detecting a natural phenomenon, such as DNA or SNPs associated with cancer.²⁴⁴

I am informed by counsel that the patent-eligibility determination requires more than determining if the claims “involve” an ineligible concept. Rather, claims may be eligible for patenting if they recite a method for achieving a new and useful result.

216. In my opinion, the Asserted Claims of the '035 Patent are directed not to a natural phenomenon, but rather to an inventive combination of specific laboratory techniques, each of which requires significant human effort to perform and which involve modifying natural-occurring products as part of the process. Although these techniques were previously known in the art, their combination as recited in the claims represents a novel and innovative improvement over earlier methods of sample preparation that achieve superior results. I discuss the advantages of the claimed methods in greater detail *infra* with respect to step two of the eligibility analysis.

217. Notably, when performed in combination as recited in the Asserted Claims of the '035 Patent, the claimed techniques permit identification of 25-2,000 SNPs associated with cancer using cfDNA, a DNA source that the art recognized as highly challenging to manipulate successfully.²⁴⁵ This claimed method therefore represents a new and useful method of sample preparation that can be used to yield a tangible and useful result. This result is only achieved by the combination of specific laboratory techniques that, together, represent a new and useful method of preparing cfDNA. The Van Ness

²⁴⁴ See Van Ness Decl. at ¶¶ 341-347.

²⁴⁵ See Section II.

Decl. does not address the new, useful result achieved by the claimed combination. Nor does Dr. Van Ness discuss certain specific limitations recited in the claims, which I consider *infra*.

218. First, the Van Ness Declaration does not address that, in the '035 Patent preamble, the Asserted Claims of the '035 Patent expressly recite that they involve “[a] method for amplifying and sequencing DNA,” not “detecting.”²⁴⁶ I believe this limitation should be considered at least because the Asserted Claims recite these specific techniques in order to prepare the cfDNA sample. Detection is not a recited step anywhere in the claimed methods. I discuss the claimed steps in turn *infra*.

219. Second, the first-recited step of the Asserted Claims of the '035 Patent requires performing two different steps, each of which depends upon human intervention and effort. Specifically, it recites [1] tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products, wherein [2] the isolated cell-free DNA is isolated from a blood sample.²⁴⁷ To perform the method, cfDNA must be isolated and then tagged with one or more universal tail adaptors. This approach generates tagged products that are not found anywhere in nature.

220. Third, the Asserted Claim of the '035 Patent recite a specific protocol for amplifying cfDNA. This step requires specific technical approaches, each of which requires significant human effort to design and implement. Specifically, the claims require

²⁴⁶ See Van Ness Decl. at ¶¶ 341-382, where the preamble is not mentioned at all.

²⁴⁷ See '035 Patent at 249:44-49.

[1] amplifying the tagged products one or more times to generate final amplification products, wherein [2] one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume, [3] wherein one of the amplifying steps introduces a barcode and one or more sequencing tags.²⁴⁸ The products of this amplification step, which are barcoded and contain sequencing tag(s), are not found anywhere in nature.

221. Fourth, the Asserted Claims of the '035 Patent recite sequencing the plurality of SNP loci on cfDNA by conducting massively parallel sequencing on the final amplification products. Like the other recited preparation steps, the massively parallel sequencing step requires significant human effort to implement and does not occur in nature. Nor is massively parallel sequencing simply “sequencing,” as described *supra*. It requires certain sequencing equipment to perform and entails sequencing on a very large scale—millions or billions of reads per each run of the instrument.

222. Accordingly, from my review of the Asserted Claims of the '035 Patent, in my opinion, their character as a whole is directed to eligible subject matter—a new and useful method for sample preparation by amplifying and sequencing cfDNA using a combination of specific laboratory techniques. Manipulating SNP-containing tumor-associated cfDNA in this way would have been particularly challenging for reasons explained *supra*.²⁴⁹ The combination of recited techniques transform DNA into artificial

²⁴⁸ *Id.* at 249:50-57.

²⁴⁹ *See* Sections II.

products with at least one universal tail adaptor, a barcode, and one or more sequencing tags—products that are not found anywhere in nature. They are not, as Dr. Van Ness opines, directed to natural phenomenon or any other ineligible subject matter.²⁵⁰

223. In reaching his contrary conclusions, Dr. Van Ness discusses other patents from separate proceedings.²⁵¹ I disagree with his assessment of these non-asserted patents at least because those patents recite different limitations.. The Van Ness Declaration fails to offer any reasoned basis to equate their recited combination to the combination recited in the Asserted Claims of the '035 Patent. Dr. Van Ness also discusses a claim amendment during prosecution concerning sequencing SNPs “associated with cancer.”²⁵² This does not change my view as, *e.g.*, sequencing cancer-associated SNPs represents a new and useful result that Dr. Van Ness acknowledges was not found in the prior art [*e.g.*, Chuu (2012)²⁵³] as the '035 Patent claims were allowable over Chuu (2012).

224. For all the aforementioned reasons, in my opinion the Asserted Claims of the '035 Patent, when considered as a whole, are directed to patent-eligible subject matter and step two of the patent-eligibility analysis is unnecessary. Even under an analysis of step two, the Asserted Claims of the '035 Patent should still be found patent-eligible, as explained *infra*.

²⁵⁰ See Van Ness Decl. at § XI.

²⁵¹ *Id.* at ¶¶ 345-346.

²⁵² *Id.* at ¶ 344.

²⁵³ See Chuu & Rava, Methods of fetal abnormality detection, U.S. Patent 8,318,430 (2012) (“Chuu (2010)”).

(ii) *The '035 Patent Claims, Considered Individually and In Combination, Are Not Routine or Conventional*

225. The Van Ness Declaration opines that certain aspects of the claimed methods of the '035 Patent—tagging, amplification, and sequencing—were routine and conventional.²⁵⁴ Even if, considered independently and in isolation, these general techniques may have been known in the art as of 2011, this is not to say, however, that these methods were routine or conventional, particularly in the context of working with ctDNA. They were not, as explained *supra*.²⁵⁵

226. In any event, in my understanding, even if the recited limitations were routine, conventional, or well-understood that would not be sufficient to show patent ineligibility, as the claim limitations must be considered both individually and as their ordered combination. The Van Ness Declaration fails to consider the latter—whether the '035 Patent limitations are well-understood, routine, or conventional in their ordered combinations. In my opinion, these claim limitations when considered in their ordered combination are not well-understood, routine, or conventional. In consequence, the Asserted Claims of the '035 Patent should be deemed patent-eligible if the Court reaches step two of the eligibility analysis.

227. When considered as an ordered combination, the method recited in the Asserted Claims of in the '035 Patent is not routine or conventional at least because the

²⁵⁴ See Van Ness Decl. at ¶¶ 348-381.

²⁵⁵ See Section II.

method's ordered steps recite improved, inventive processes for sample preparation. This ordered combination improves upon prior art approaches in several respects, as described *infra*.

228. First, as the '035 Patent explains, certain problems in multiplex amplification were not addressed by the prior art. These problems become more pronounced when the number of reactions are performed in a single volume increases. One example is the formation of “primer-dimers”—PCR primers that bind to each other rather than their targets—which may reduce the amplification of certain targets. Another example is “allelic bias,” where some targets are amplified more efficiently than others, skewing the representative amounts of cancer-associated versus non-cancer-related cfDNA present in the sample. As exemplified *infra*, the '035 Patent identifies various limitations in prior art techniques.

Use of multiplex PCR can significantly simplify experimental procedures and shorten the time required for nucleic acid analysis and detection. ***However, when multiple pairs are added to the same PCR reaction, non-target amplification products may be generated, such as amplified primer dimers.*** The risk of generating such products increases as the number of primers increases. These non-target amplicons significantly limit the use of the amplified products for further analysis and/or assays. ***Thus, improved methods are needed to reduce the formation of non-target amplicons during multiplex PCR.***²⁵⁶

Currently, performing multiplex PCR reactions of more than 5 to 10 targets presents a major challenge and is often hindered by primer side products, such as primer dimers, and

²⁵⁶ See '035 Patent at 3:4-14 (emphases added).

other artifacts. When detecting target sequences using microarrays with hybridization probes, primer dimers and other artifacts may be ignored, as these are not detected. ***However, when using sequencing as a method of detection, the vast majority of the sequencing reads would sequence such artifacts and not the desired target sequences in a sample.*** Methods described in the prior art used to multiplex more than 50 or 100 reactions in one reaction volume followed by sequencing will typically result in more than 20%, and often more than 50%, in many cases more than 80% and in some cases more than 90% off-target sequence reads.²⁵⁷

In general, to perform targeted sequencing of multiple (n) targets of a sample (greater than 50, greater than 100, greater than 500, or greater than 1,000), ***one can split the sample into a number of parallel reactions that amplify one individual target.*** This has been performed in PCR multiwell plates or can be done in commercial platforms such as the FLUIDIGM ACCESS ARRAY (48 reactions per sample in microfluidic chips) or DROPLET PCR by RAIN DANCE TECHNOLOGY (100 s to a few thousands of targets). Unfortunately, these split-and-pool methods are problematic for samples with a limited amount of DNA, as there is often not enough copies of the genome to ensure that there is one copy of each region of the genome in each well.²⁵⁸

Disclosed herein are methods that permit the targeted amplification of over a hundred to tens of thousands of target sequences (e.g., SNP loci) from a nucleic acid sample such as genomic DNA obtained from plasma. ***The amplified sample may be relatively free of primer dimer products and have low allelic bias at target loci.*** If during or after amplification the products are appended with sequencing compatible adaptors, analysis of these products can be performed by sequencing.²⁵⁹

²⁵⁷ *Id.* at 85:5-20 (emphases added).

²⁵⁸ *Id.* at 85:21-33 (emphasis added).

²⁵⁹ *Id.* at 86:2-10 (emphasis added).

229. The claimed methods of the '035 Patent address these problems in inventive ways that were not routine or conventional in 2011. Notably, the claimed approach recited in the Asserted Claims of the '035 Patent would have been technically challenging as of the priority date. As one of ordinary skill in the art would have recognized, a mixture containing cfDNA derived from both tumor cells and non-cancerous cells presents particular challenges in multiplex amplification suitable for subsequent massively parallel sequencing. This is due at least in part to the scarcity of tumor-derived cfDNA in the sample. These challenges are magnified as the number of SNPs amplified within the single reaction mixture increases. In overcoming these technical challenges, the '035 Patent teaches that its methods can be used to prepare small amounts of cancer-associated cfDNA, which are challenging to manipulate, for example, because they are fragmented.²⁶⁰

Primer tails may improve the detection of fragmented DNA from universally tagged libraries. If the library tag and the primer-tails contain a homologous sequence, hybridization can be improved (for example, melting temperature (T_M) is lowered) and primers can be extended if only a portion of the primer target sequence is in the sample DNA fragment.²⁶¹

230. Second, the specific claimed approach recited in the Asserted Claims of the '035 Patent also represents an improvement over prior art techniques because amplifying multiple targeted loci together, when done successfully as the '035 Patent describes, is efficient and provides more comprehensive genetic information than amplifying only one

²⁶⁰ See Section II, *supra*.

²⁶¹ See '035 Patent at 94:51-57 (emphasis added).

or a few targets. It reduces the time and operational complexity in a method that requires processing and sequencing up to thousands of SNPs.

Improved PCR amplification methods have also been developed that minimize or prevent interference due to the amplification of nearby or adjacent target loci in the same reaction volume (such as part of the sample multiplex PCR reaction that simultaneously amplifies all the target loci) ... ***These methods can be used to simultaneously amplify nearby or adjacent target loci, which is faster and cheaper than having to separate nearby target loci into different reaction volumes*** so that they can be amplified separately to avoid interference.²⁶²

Disclosed herein are methods that permit the targeted amplification of over a hundred to tens of thousands of target sequences (e.g., SNP loci) from a nucleic acid sample such as genomic DNA obtained from plasma. ***The amplified sample may be relatively free of primer dimer products and have low allelic bias at target loci.*** If during or after amplification the products are appended with sequencing compatible adaptors, analysis of these products can be performed by sequencing.²⁶³

The use of tags on the primers may reduce amplification and sequencing of primer dimer products.²⁶⁴

In an embodiment, a 10,000-plex PCR assay pool is created such that reverse primers have tails corresponding to the required reverse sequences required by a high throughput sequencing instrument. After amplification with the first 10,000-plex assay, a subsequent PCR amplification may be performed using a another 10,000-plex pool having partly nested forward primers (e.g. 6-bases nested) for all targets and a reverse primer corresponding to the reverse sequencing tail included in the first round. ***This subsequent round of partly nested amplification with just one target specific primer and***

²⁶² *Id.* at 101:29-41 (emphasis added).

²⁶³ *Id.* at 86:2-10 (emphasis added).

²⁶⁴ *Id.* at 55:11-12 (emphasis added).

a universal primer limits the required size of the assay, reducing sampling noise, but greatly reduces the number of spurious amplicons. The sequencing tags can be added to appended ligation adaptors and/or as part of PCR probes, such that the tag is part of the final amplicon.²⁶⁵

231. Third, the claimed approach recited in the Asserted Claims of the '035 Patent is also advantageous because it has the additional benefit of avoiding dividing the plasma sample into individual reactions. Dividing a cfDNA sample may lead to biased results when, for example, the sample contains very low and/or variable levels of tumor-associated cfDNA.

For samples where either the overall sample or some subpopulation of DNA molecules is limited, splitting the sample would introduce statistical noise. In an embodiment, a small or limited quantity of DNA may refer to an amount below 10 µg, between 10 and 100 µg, between 100 µg and 1 ng, between 1 and 10 ng, or between 10 and 100 ng. Note that while this method is particularly useful on small amounts of DNA where other methods that involve splitting into multiple pools can cause significant problems related to introduced stochastic noise, this method still provides the benefit of minimizing bias when it is run on samples of any quantity of DNA.²⁶⁶

Note that this approach could be used to perform targeted amplification in a manner that would result in low levels of allelic bias for 50-500 loci, for 500 to 5,000 loci, for 5,000 to 50,000 loci, or even for 50,000 to 500,000 loci. In an embodiment, the primers carry partial or full length sequencing compatible tags.²⁶⁷

²⁶⁵ *Id.* at 91:4-18 (emphasis added).

²⁶⁶ *Id.* at 84:51-62 (emphasis added).

²⁶⁷ *Id.* at 86:54-60 (emphasis added).

232. In addition to my opinions *supra*, I further note that the prior art cited by Dr. Van Ness under Section 103 does not show that any reference teaches each and every limitation of the claims, arranged as in the claim. The fact that even combinations of references do not teach or suggest the claimed methods as recited further supports my opinion that the ordered combination of the claims was not routine, conventional, or well-understood in the art.

VIII. THE NATERA PATENTS ARE VALID UNDER SECTION 112

233. The Van Ness Declaration additionally opines that the Natera Patents are invalid under Section 112 for lack of adequate written description.²⁶⁸ I disagree with the bases Dr. Van Ness contends are invalidating at least for the reasons explained *infra*. I reserve the right to offer additional opinions at another time.

234. Notably, the Van Ness Declaration does not raise any challenge to the Asserted Claims under either an indefiniteness or lack of enablement theory under Section 112. I therefore do not address these grounds herein. To the extent Dr. Van Ness raises such a challenge in the future, I reserve the right to offer opinions in rebuttal.

A. The '454 Patent Adequately Describes Whole Genome Sequencing

235. The Van Ness Declaration contends that there is no description of “whole genome sequencing” in the '454 Patent.²⁶⁹ This is incorrect and I disagree with Dr. Van

²⁶⁸ See Van Ness Decl. at ¶¶ 404-426.

²⁶⁹ *Id.* at ¶¶ 405-407.

Ness. Further, the Van Ness Declaration’s analysis is flawed in several respects, as described *infra*.

236. First, I note that his belief is informed by his review of Jamal-Hanjani Thesis (2015)²⁷⁰ and the testimony of a Natera witness, Bernhard Zimmermann.²⁷¹ It is my understanding from counsel that the adequacy of a patent’s written description is determined based on the written description within the four corners of that patent, and from the perspective of one of ordinary skill in the art. The Jamal-Hanjani Thesis (2015) is a separate document and not part of the patent’s four corners—for example, not incorporated by reference. The Van Ness Declaration does not contend otherwise. Dr. Zimmermann’s testimony is also not part of the patent disclosure. Therefore, to the extent that the opinions expressed in the Van Ness Declaration are founded on these extrinsic sources of information, it is my understanding that Dr. Van Ness has misapplied the relevant legal principles governing written description.

237. Second, the ’454 Patent explicitly uses the words “whole genome sequencing” to describe experimental results reported in the ’454 Patent. I reproduce this teaching *infra*. In the accompanying Figure 51B, the ’454 Patent reports data on certain

²⁷⁰ See Dkt. 118-1, Jamal-Hanjani, *The role of intratumour heterogeneity and chromosomal instability in cancer*, University College London, PhD Supervisor: Professor Charles Swanton; A thesis submitted for the degree of Doctor of Philosophy, University College London (“Jamal-Hanjani Thesis (2015)”).

²⁷¹ See Van Ness Decl. at ¶¶ 406-407.

genes, which could be captured either by whole genome sequencing or by whole exome sequencing.

FIG. 51A represents the histological finding/history for primary lung tumors analyzed for clonal and subclonal tumor heterogeneity. FIG. 51B is a table of the VAF identities of the biopsied lung tumors by *whole genome sequencing* and assaying by AmpliSEQ.²⁷²

Sample	Gene	Chr	ChrStart	VAF R1	VAF R2	VAF R3
L12	BRIP1	chr17	59924572	14	6	8
L12	CARS	chr11	3062181	22	11	16
L12	CIC	chr19	42797381	0	0	7
L12	CYFIP1	chr15	22940733	0	6	0
L12	FAT1	chr4	187519147	8	4	0
L12	KDM6A	chrX	44921898	10	5	0
L12	MLLT4	chr6	168347475	9	3	0
L12	NFE2L2	chr2	178098801	69	31	50
L12	RASA1	chr5	86642517	7	0	0
L12	TP53	chr17	7578406	23	9	17
L12	TP53	chr17	7578190	22	8	16
L13	EGFR	chr7	55241708	21	25	55
L13	EGFR	chr7	55242511	20	17	48
L13	HERC4	chr10	69793756	0	3	6
L13	JAK2	chr9	5022084	0	11	7
L13	KMT2C	chr7	151947008	0	0	4
L13	MSH2	chr2	47693816	5	0	11
L13	MTOR	chr1	11292495	2	0	3
L13	PLCG2	chr16	81942036	5	0	6
L13	TP53	chr17	7579509	7	4	16
L15	ALK	chr2	29940530	6	2	
L15	GABRG1	chr4	46060315	13	0	
L15	KDM6A	chrX	44922755	18	6	
L15	MLL2	chr12	49443815	5	0	
L15	ROS1	chr6	117687379	10	3	
L15	SLC39A4	chr8	145638322	14	0	
L15	TP53	chr17	7578254	19	3	
L15	ZFHX4	chr8	77776735	13	5	
L15	ZMYM4	chr1	35827319	14	2	
L17	BRCA2	chr13	32914959	16	28	0
L17	KRAS	chr12	25398284	13	22	0
L17	NF1	chr17	29653134	20	25	0
L17	NF1	chr17	29528088	0	0	20
L17	PAX8	chr2	113984793	9	0	0
L17	TP53	chr17	7577610	0	0	16
L17	TP53	chr17	7577079	20	35	0
L17	TRIM67	chr1	231299607	0	50	0
L17	TRIP11	chr14	92471631	7	0	0

FIG. 51B

238. Separately, the '454 Patent also refers to whole exome sequencing in Example 13, but it does not state that only whole exome sequencing was performed. At most, the '454 Patent's disclosures reflect an ambiguity or typographical error (*e.g.*, referring to whole exome when whole genome was actually performed).

²⁷² See '454 Patent at 41:57-61 (emphasis added).

239. In any event, this ambiguity would not have taught one of ordinary skill in the art that the inventors did not possess the use of whole genome sequencing in the context of the claimed methods. To the contrary, whole exome sequencing is a modification of whole genome sequencing, in which specific portions of the genome (the protein-coding portions, called exons) are targeted and enriched. Thus, by the '454 Patent's plain language, one of ordinary skill in the art would have understood that the named inventors possessed the use of both whole genome sequencing and whole exome sequencing to achieve the claimed methods.

240. Third, the Van Ness Declaration does not dispute that whole genome sequencing was well-known in the art in 2014-2015. On the contrary, in the Van Ness Declaration's opinions regarding Section 101, Dr. Van Ness asserts that "[w]hole genome sequencing and whole exome sequencing were well known to a POSA."²⁷³ Dr. Van Ness points to multiple sources for the proposition that whole genome sequencing was a routine and conventional technique in the 2014-2015 time period and acknowledges that "the '454 Patent cites to a wealth of prior art publications describing routine, standard, gene sequencing techniques known in the art, including whole genome sequencing."²⁷⁴

241. As to the general technique of whole genome sequencing, I agree with the Van Ness Declaration that whole genome sequencing was well known in the art before 2014-2015. As Dr. Van Ness admits, multiple prior art references cited in the '454 Patent

²⁷³ See Van Ness Decl. at ¶ 318.

²⁷⁴ *Id.* at ¶ 320.

describe the use of this technique for various purposes.²⁷⁵ Even looking beyond the '454 Patent, other prior art describes the use of this technique, further establishing that it was known in the art. As one example, in 2010, I published Metzker (2010), which is a review discussing the use of whole genome sequencing for various purposes. I excerpt *infra* Table 1²⁷⁶ from that review that illustrates certain early “biological applications” that could be achieved using whole genome sequencing and whole exome sequencing approaches. These biological applications had been performed using commercially-available next-generation sequencing technologies.

²⁷⁵ *Id.*

²⁷⁶ *See* Metzker (2010) at Table 1.

Table 1 | Comparison of next-generation sequencing platforms

Platform	Library/ template preparation	NGS chemistry	Read length (bases)	Run time (days)	Gb per run	Machine cost (US\$)	Pros	Cons	Biological applications
Roche/454's GS FLX Titanium	Frag, MP/ emPCR	PS	330*	0.35	0.45	500,000	Longer reads improve mapping in repetitive regions; fast run times	High reagent cost; high error rates in homo- polymer repeats	Bacterial and insect genome <i>de novo</i> assemblies; medium scale (<3 Mb) exome capture; 16S in metagenomics
Illumina/ Solexa's GA _{II}	Frag, MP/ solid-phase	RTs	75 or 100	4 [†] , 9 [‡]	18 [†] , 35 [‡]	540,000	Currently the most widely used platform in the field	Low multiplexing capability of samples	Variant discovery by whole-genome resequencing or whole-exome capture; gene discovery in metagenomics
Life/APG's SOLiD 3	Frag, MP/ emPCR	Cleavable probe SBL	50	7 [†] , 14 [‡]	30 [†] , 50 [‡]	595,000	Two-base encoding provides inherent error correction	Long run times	Variant discovery by whole-genome resequencing or whole-exome capture; gene discovery in metagenomics
Polonator G.007	MP only/ emPCR	Non- cleavable probe SBL	26	5 [‡]	12 [‡]	170,000	Least expensive platform; open source to adapt alternative NGS chemistries	Users are required to maintain and quality control reagents; shortest NGS read lengths	Bacterial genome resequencing for variant discovery
Helicos BioSciences HeliScope	Frag, MP/ single molecule	RTs	32*	8 [†]	37 [†]	999,000	Non-bias representation of templates for genome and seq-based applications	High error rates compared with other reversible terminator chemistries	Seq-based methods
Pacific Biosciences (target release: 2010)	Frag only/ single molecule	Real-time	964*	N/A	N/A	N/A	Has the greatest potential for reads exceeding 1 kb	Highest error rates compared with other NGS chemistries	Full-length transcriptome sequencing; complements other ressequencing efforts in discovering large structural variants and haplotype blocks

242. Fourth, it is my understanding that a patent does not need to describe an invention in the exact same words as recited in the claim as long as one of ordinary skill in the art would have understood that the named inventors were in possession of that aspect of the invention. From my review of the '454 Patent, it describes the use of whole genome sequencing in different words than simply calling out the technique's specific name. In other words, the '454 Patent describes familiar applications of whole genome sequencing which one of ordinary skill in the art would have recognized as such despite not referring to those applications as involving whole genome sequencing.

243. For example, the '454 Patent describes a technique called “long read sequencing.”²⁷⁷ As one application of this technique, the '454 Patent describes “phasing,” which is a method to sequence long stretches of DNA on the same chromosome.²⁷⁸ “Phasing refers to the act of determining the haplotypic genetic data of an individual given unordered, diploid (or polyploidy) genetic data.”²⁷⁹ The '454 Patent describes “Exemplary Methods for Phasing Genetic Data” across several columns.²⁸⁰ These techniques form “long haplotype contigs” of DNA—*i.e.*, reassembling the genome from the phased sequencing data. “After sequencing, the computational steps involve ... assembling the fragments at their overlapping heterozygous SNVs into haplotype blocks, and phasing the blocks statistically based on a phased reference panel and producing long haplotype contigs.”²⁸¹ Such approaches could be performed, for example, using Illumina’s Moleculo Technology described in the ‘454 Patent, which one of ordinary skill in the art would have

²⁷⁷ See '454 Patent at 116:20-35; 112:50-56; *see also* Ex. 9, Kuleshov *et al.*, *Whole-genome haplotyping using long reads and statistical methods*, NATURE BIOTECHNOLOGY 32:261-266 (2014) (“Kuleshov (2014)”) (“**Here we describe statistically aided, long-read haplotyping (SLRH)**, ... For a human sample, as little as 30 Gbp of additional sequencing data are needed to phase genotypes identified by **50× coverage whole-genome sequencing**”) at Abstract (emphases added); *see also* Ex. 10, GenomeWeb article dated March 5, 2014 describing that “Stanford University was an early-access user of Moleculo technology, and researchers there detailed in a *Nature Biotechnology* publication last week ... Volodymyr Kuleshov, a former consultant with Moleculo, now a member of Mike Snyder’s Stanford laboratory and also the lead author of the paper.”

²⁷⁸ *Id.* at 115:7-34; 116:20-123:3.

²⁷⁹ *Id.* at 32:35-37.

²⁸⁰ *Id.* at 123:3-127:46.

²⁸¹ *Id.* at 116:20-35.

understood can perform whole genome sequencing.²⁸² “In some embodiments, a haplotype of an individual is determined by long read sequencing, such as by using the Molecule Technology developed by Illumina.”²⁸³

244. The ’454 Patent describes other techniques that could perform whole genome sequencing, including whole genome amplification (WGA) methods.

In some embodiments, whole genome application [*sic*] (WGA) is used to amplify a nucleic acid sample. There are a number of methods available for WGA: ligation-mediated PCR (LM-PCR), degenerate oligonucleotide primer PCR (DOP-PCR), and multiple displacement amplification (MDA).²⁸⁴

One of ordinary skill in the art would have understood that these descriptions implicate the use of whole genome sequencing, despite not being described using that specific phrase.

245. I understand from his deposition that Dr. Van Ness did not consider the ’454 Patent’s disclosure of long-read sequencing, described *supra*, in rendering his opinions. I note Dr. Van Ness agrees with me that one of ordinary skill in the art would have understood that long-read sequencing can achieve whole genome sequencing.

Q. Okay. Thank you. Have you heard the concept of long-read sequencing?

A. *Yes.*

Q. You don’t discuss long-read sequencing in your declaration, correct?

²⁸² See Kuleshov (2014) at Abstract.

²⁸³ ’454 Patent at 116:20-22.

²⁸⁴ *Id.* at 96:30-34.

A. I don't see any reference to long-read – I'm not sure if I saw a reference to long-read sequencing. I did not discuss it. I discuss next-gen sequencing. Long-read sequences -- sequencing is one of the concepts of next-gen sequencing.

Q. You agree that persons of skill in the art would understand that long-read sequencing can achieve whole genome sequencing?

A. *It can achieve it.*

Q. And do you have any understanding of whether the '454 patent describes long-read sequencing? Did you consider that?

A. *I did not consider that.*²⁸⁵

246. Dr. Van Ness also agreed that with my opinions regarding phasing and determining a haplotype, but did not consider the '454 Patent's disclosures of those techniques in rendering his opinions.

Q. You would agree that phasing is performed using whole genome sequencing?

A. It's one of the components of analyzing the data and coming up with a whole genome sequence.

Q. And do you have any understanding of whether the '454 patent describes phasing?

A. I don't recall.

Q. Did you consider any discussion in the '454 patent of phasing in forming your opinions regarding written description of whole genome sequencing?

²⁸⁵ See Van Ness Tr. at 269:17-270:14 (emphases added).

A. *I did not consider that.*

Q. Did you consider any discussion in the '454 patent of phasing in forming your opinions regarding written description of whole genome sequencing?

A. *I did not consider that.*

Q. Determining a haplotype of an individual is a way of -- involves whole genome sequencing, correct?

A. It can. It's not the only way.

Q. But a person of skill in the art would understand that whole genome sequencing can be used to determine the haplotype of an individual, correct?

A. It can.

Q. And you -- you didn't consider determining haplotypes of individuals in formulating your written description opinions regarding the '454 patent?

A. I -- it was not an issue of -- that I considered to reflect any kind of lack of description, so I -- *I did not consider it.*²⁸⁶

247. From these disclosures, one of ordinary skill in the art would have recognized that the named inventors were in possession of whole genome sequencing to perform the claimed methods. This is in addition to the explicit reference to "whole genome sequencing" in the '454 Patent. as described *supra*.

²⁸⁶ *Id.* at 271:5-272:11 (emphases added).

B. The '454 and '035 Patents Adequately Describe the Claimed Methods

248. The Van Ness Declaration asserts that the Natera Patent claims are also invalid for failure to describe techniques of PCR amplification without the use of techniques for selecting primers, *e.g.*, to avoid primer dimers.²⁸⁷ Relatedly, the Van Ness Declaration contends that Dr. Van Ness does not “believe there is any embodiment that discloses the elements of the claimed inventions of the '454 or '035 Patents as arranged in the claims.”²⁸⁸ I disagree with his contentions as explained *supra*. In my opinion, one of ordinary skill in the art would have recognized that the named inventors of the Natera Patents were in possession of the claimed methods as of the Natera Patents’ respective priority dates.

(i) *The Patents Adequately Describe Methods that Do Not Require Selecting Primers to Avoid Primer Dimers*

249. As an initial matter, I note that it is technically feasible to perform multiplex PCR without taking steps to avoid primer dimers, as usable sequencing data can be obtained even if primer dimers are generated. As the '035 Patent notes, only a small number of “bad” primers are responsible for forming the majority of the observed primer dimers in any reaction—thus, most amplifications will avoid primer dimer formation without involving primer selection approaches.²⁸⁹ “The present invention is based in part

²⁸⁷ A “primer dimer” occurs when primers intended to amplify a target DNA sequence instead bind and amplify each other; *see also* Van Ness Decl. at ¶¶ 409-426.

²⁸⁸ *Id.* at ¶ 414.

²⁸⁹ *See* '035 Patent at 48:30-47.

on the surprising discovery that *often only a relatively small number of primers* in a library of primers *are responsible for a substantial amount of the amplified primer dimers* that form during multiplex PCR reactions.”²⁹⁰

250. Even in the situation where the correctly-amplified (targeted) product represents a minor fraction of the amplification and the (off-target) primer dimers represent the major fraction, next-generation sequencing technology permits excluding primer dimers by size selection in the library preparation workflow. For example, the primer dimers are comparatively small relative to the targeted amplicon, they can be excluded from analysis on the basis of size. One relatively simple method for doing this that was known to one of ordinary skill in the art was to gel-purify or SPRI purify the amplicons.²⁹¹

251. Indeed, the ’454 Patent explains that avoiding primer dimers is merely an optional component of the claimed methods, albeit an advantageous one.

Exemplary Primer Design Methods

If desired, multiplex PCR may be performed using primers with a decreased likelihood of forming primer dimers. In particular, highly multiplexed PCR can often result in the production of a very high proportion of product DNA that results from unproductive side reactions such as primer dimer formation. *In an embodiment, the particular primers that are most likely to cause unproductive side reactions may be removed from the primer library* to give a primer library that

²⁹⁰ *Id.* at 46:37-41 (emphases added).

²⁹¹ *See* Meyer (2010) at 5 “Carboxyl-coated magnetic beads (SPRI beads) are ideally suited for reaction purification in a 96-well plate setup. However, under the conditions described here, *SPRI purification does not retain molecules shorter than 100-150 bp.*” (emphasis added.)

will result in a greater proportion of amplified DNA that maps to the genome.²⁹²

252. Similarly, one of ordinary skill in the art would have understood that the '035 Patent teaches that primers do not need to be designed to avoid primer dimers, although it could be beneficial in some instances to do so. The '035 Patent explains, for example that “[i]n *some embodiments*, the test primers are selected from a library of candidate primers based at least in part on the ability of the candidate primers to form primer dimers.”²⁹³ “The amplified sample *may* be relatively free of primer dimer products and have low allelic bias at target loci.”²⁹⁴

253. Consistent with this statement, the '035 Patent describes methods of performing PCR that do not include designing primers to avoid the formation of primer dimers although it would be desirable and standard practice in the art to do so.²⁹⁵ For example, the '035 Patent describes that “using lower primer concentrations and much longer annealing times than normal increases the likelihood that the primers hybridize to the target loci instead of hybridizing to each other and forming primer dimers.”²⁹⁶ In another amplification strategy, the primers’ “annealing temperature for the reaction

²⁹² See '454 Patent at 105:48-58 (emphases added).

²⁹³ See '035 Patent at 11:29-31.

²⁹⁴ *Id.* at 86:5-7 (emphasis added).

²⁹⁵ *Id.* at 48:30-47.

²⁹⁶ *Id.* at 46:51-55.

conditions is greater than a melting temperature.”²⁹⁷ The ’035 Patent also teaches other, different methods of improving the quality of its amplification results, for example, by avoiding allelic bias.²⁹⁸ “A number of methods are described herein that may be used to preferentially enrich a sample of DNA at a plurality of loci in a way that minimizes allelic bias.”²⁹⁹ Dr. Van Ness also testified that it would be feasible to avoid allelic bias.

Q. What if you know that there are certain target loci that lead to allelic bias and you design your PCR multiplex assay to avoid those loci? Would that be one way to avoid allelic bias?

A. Yeah, that is sort of a – I mean, hypothetically, sure, if you wanted to eliminate certain targeted sequences *because you couldn’t accurately develop the allelic representation, you could just avoid them just by not including primers to amplify those particular sequences*. Sure, hypothetically.³⁰⁰

254. The Natera Patents therefore contemplate that primer dimers can be present at some level. This is also reflected in the following disclosures.

In various embodiments, less than 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% of the amplified products are primer dimers.³⁰¹

In some embodiments, (i) less than 60% of the amplified products are primer dimers and at least 40% of the amplified products are target amplicons, (ii) less than 40% of the amplified products are primer dimers and at least 60% of the

²⁹⁷ *Id.* at 4:30-31.

²⁹⁸ *Id.* at 86:2-10.

²⁹⁹ *Id.* at 197:52-54.

³⁰⁰ *See* Van Ness Tr. at 276:3-15 (objection omitted, emphasis added).

³⁰¹ *See* ’454 Patent at 98:18-21.

amplified products are target amplicons, (iii) less than 20% of the amplified products are primer dimers and at least 80% of the amplified products are target amplicons, (iv) less than 10% of the amplified products are primer dimers and at least 90% of the amplified products are target amplicons, or (v) less than 5% of the amplified products are primer dimers and at least 95% of the amplified products are target amplicons.³⁰²

255. Moreover, any avoidance of primer dimers during the 2011-2015 time period was not novel or inventive. By 2011, one of ordinary skill in the art would have been familiar with multiplex PCR amplification. Even in well-functioning assays at that time, primer dimers would have been likely to occur. Although it would not have been necessary to design primers to avoid primer dimers, there were known methods to do so.

(ii) *The Natera Patents Contain Examples of the Claimed Methods*

256. I am informed by counsel that the adequacy of written description does not require any particular form of disclosure, and that, for example, examples are not required. The Natera Patents adequately describe the claimed methods, including with the use of Examples. For example, the Asserted Claims of the '454 Patent recite the following.

Claim 1: A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:
 performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;
 performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein

³⁰² See '035 Patent at 22:3-14.

the target loci are amplified together in the same reaction volume; and
 sequencing the amplicons to obtain sequence reads, and
 detecting one or more of the tumor-specific SNV mutations
 present in the cell-free DNA from the sequence reads,
 wherein the sequencing has a depth of read of at least
 50,000 per target locus.

Claim 8: The method of claim 1, wherein the targeted multiplex amplification amplifies 20 to 50 target loci each encompassing a different tumor-specific SNV mutation.

Claim 11: The method of claim 1, wherein the method further comprises performing barcoding PCR prior to the sequencing.

257. Such limitations are described throughout the '454 Patent, including the legend of Fig. 51B described *supra*. Exemplary disclosures from the specification are reproduced *infra*, including from the '454 Patent's description of "Exemplary Sample Preparation Methods."

Exemplary Sample Preparation Methods

In some embodiments, the method includes isolating or purifying the DNA and/or RNA.³⁰³

In some embodiments, whole genome application (WGA) is used to amplify a nucleic acid sample. There are a number of methods available for WGA: ligation-mediated PCR (LM-PCR), degenerate oligonucleotide primer PCR (DOP-PCR), and multiple displacement amplification (MDA). In LM-PCR, short DNA sequences called adapters are ligated to blunt ends of DNA.³⁰⁴

In some embodiments, PCR (referred to as mini-PCR) is used to generate very short amplicons (U.S. application Ser. No. 13/683,604, filed Nov. 21, 2012, U.S. Publication No.

³⁰³ See '454 Patent at 95:41-43.

³⁰⁴ *Id.* at 96:30-36.

2013/0123120, U.S. application Ser. No. 13/300,235, filed Nov. 18, 2011, U.S. Publication No 2012/0270212, filed Nov. 18, 2011, and U.S. Ser. No. 61/994,791, filed May 16, 2014, which are each hereby incorporated by reference in its entirety).³⁰⁵

In some embodiments, multiplex PCR is used. In some embodiments, the method of amplifying target loci in a nucleic acid sample involves (i) contacting the nucleic acid sample with a library of primers that simultaneously hybridize to least 100; 200; 500; 750; 1,000; 2,000; 5,000; 7,500; 10,000; 20,000; 25,000; 30,000; 40,000; 50,000; 75,000; or 100,000 different target loci to produce a reaction mixture; and (ii) subjecting the reaction mixture to primer extension reaction conditions (such as PCR conditions) to produce amplified products that include target amplicons. In some embodiments, at least 50, 60, 70, 80, 90, 95, 96, 97, 98, 99, or 99.5% of the targeted loci are amplified. In various embodiments, less than 60, 50, 40, 30, 20, 10, 5, 4, 3, 2, 1, 0.5, 0.25, 0.1, or 0.05% of the amplified products are primer dimers.³⁰⁶

The selective enrichment and/or amplification may involve tagging each individual molecule with different tags, molecular barcodes, tags for amplification, and/or tags for sequencing. In some embodiments, the amplified products are analyzed by sequencing (such as by high throughput sequencing) or by hybridization to an array, such as a SNP array, the ILLUMINA INFINIUM array, or the AFFYMETRIX gene chip.³⁰⁷

258. The '454 Patent also teaches that “[i]n certain embodiments, a depth of read of greater than ... 50,000, or 100,000 on the low end of the range and ... 50,000, 100,000, 250,000 or 500,000 reads on the high end, is attained in the sequencing run for each single

³⁰⁵ *Id.* at 97:13-20.

³⁰⁶ *Id.* at 98:7-21.

³⁰⁷ *Id.* at 98:35-42.

nucleotide variant position in the set of single nucleotide variant positions.”³⁰⁸ In other examples of amplifying SNV loci, for example, the inventors “observed improved DOR efficiency with increasing cycles,” and “[t]he limit of detection decreased (i.e. SNV sensitivity increased) with increasing depth of read.”³⁰⁹ Moreover, the ’454 Patent describes “exemplary methods for calculating the limit of detection for any of the methods of the invention,” including methods “used to calculate the limit of detection for single nucleotide variants (SNVs) in a tumor biopsy.”³¹⁰

259. The ’035 Patent claims are also adequately described. The Asserted Claims of the ’035 Patent recite the following.

Claim 1: A method for amplifying and sequencing DNA, comprising:
tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products, wherein the isolated cell-free DNA is isolated from a blood sample collected from a subject who is not a pregnant women;
amplifying the tagged products one or more times to generate final amplification products, wherein one of the amplification steps comprises targeted amplification of a plurality of single nucleotide polymorphism (SNP) loci in a single reaction volume, wherein one of the amplifying steps introduces a barcode and one or more sequencing tags; and
sequencing the plurality of SNP loci on the cell free DNA by conducting massively parallel sequencing on the final amplification products, wherein the plurality of SNP loci comprises 25-2,000 loci associated with cancer.

³⁰⁸ *Id.* at 68:33-39.

³⁰⁹ *Id.* at 169:57-62, citing to Figs. 55-61.

³¹⁰ *Id.* at 157:51-55, citing to Example 6.

Claim 12: The method of claim 1, wherein the one or more universal tail adaptors comprise a first universal tail adaptor and a second universal tail adaptor.

Claim 13: The method of claim 12, wherein tagging the cell free DNA comprises amplifying the cell free DNA with a first primer comprising the first universal tail adaptor and a second primer comprising the second universal tail adaptor.

260. Such limitations are adequately described at, *e.g.*, the following disclosures in the '035 Patent:

Disclosed herein are methods that permit the targeted amplification of over a hundred to tens of thousands of target sequences (e.g., SNP loci) from a nucleic acid sample such as genomic DNA obtained from plasma.³¹¹

The workflow may entail (1) extracting DNA such as plasma DNA, (2) preparing fragment library with universal adaptors on both ends of fragments, (3) amplifying the library using universal primers specific to the adaptors, (4) dividing the amplified sample "library" into multiple aliquots, (5) performing multiplex (e.g. about 100-plex, 1,000, or 10,000-plex with one target specific primer per target and a tag-specific primer) amplifications on aliquots, (6) pooling aliquots of one sample, (7) barcoding the sample, (8) mixing the samples and adjusting the concentration, (9) sequencing the sample. The workflow may comprise multiple sub-steps that contain one of the listed steps (e.g. step (2) of preparing the library step could entail three enzymatic steps (blunt ending, dA tailing and adaptor ligation) and three purification steps). Steps of the workflow may be combined, divided up or performed in different order (e.g. bar coding and pooling of samples).³¹²

³¹¹ See '035 Patent at 86:2-5.

³¹² *Id.* at 86:61-87:10.

261. The '035 Patent further describes that its methods “are especially useful when the sample of DNA is particularly small, or when it is a sample of DNA where the DNA originates from more than one individual.”³¹³ The '035 Patent further describes that its methods may be “used in the case where cells of different genetic constitution may be present in a single individual, such as with cancer.”³¹⁴

(iii) *The '035 Patent Adequately Describes Use of Universal Tail Adaptors*

262. Relatedly, Dr. Van Ness opines in conclusory fashion that the '035 Patent does not adequately describe the use of universal tail adaptors.³¹⁵ I disagree as there is adequate written description of the use of such adaptors in the '035 Patent specification. In addition to the disclosure *supra*, the '035 Patent describes these adaptors as described *infra*.

Universal Adapters, or ‘ligation adaptors’ or ‘library tags’ are DNA molecules containing a universal priming sequence that can be covalently linked to the 5-prime and 3-prime end of a population of target double stranded DNA molecules. The addition of the adapters provides universal priming sequences to the 5-prime and 3-prime end of the target population from which PCR amplification can take place, amplifying all molecules from the target population, using a single pair of amplification primers.³¹⁶

³¹³ *Id.* at 95:25-27.

³¹⁴ *Id.* at 95:32-34.

³¹⁵ *See* Van Ness Decl. at ¶ 425.

³¹⁶ *See* '035 Patent at 42:23-30.

263. In terms of using the adaptors, the '035 Patent specification teaches that “preparing the sample (e.g., the first sample) further includes appending *universal adapters* to the DNA in the sample (e.g., the first sample) and amplifying the DNA in the sample (e.g., the first sample) using the polymerase chain reaction.”³¹⁷ The universal adapters, as described, are covalently linked to the ends of the DNA strands (through known means), and as such are considered “tail” adaptors.

264. For all of these reasons, it is my opinion that one of ordinary skill in the art would have recognized that the Natera Patent inventors possessed the claimed methods, including all of the recited limitations, as of the Natera Patents’ respective priority dates. Consequently, the Asserted Claims of the Natera Patents are not invalid under Section 112 for lack of written description.

IX. INFRINGEMENT OF THE NATERA PATENTS

A. '454 Patent

265. As explained *infra*, I disagree with Dr. Van Ness’s contention that the accused RaDaR assay does not infringe the Asserted Claims of the '454 Patent.

266. As an initial matter, Dr. Van Ness’s noninfringement theory is predicated on a claim construction that deviates from the plain and ordinary meaning of the claim language. With one exception (“amplifying,” discussed *infra*)³¹⁸, Dr. Van Ness has not offered opinions on the construction of any claim terms that underlie NeoGenomics’

³¹⁷ *Id.* at 32:19-23 (emphasis added).

³¹⁸ *See* Van Ness Decl. at § VII; *see also id.* at § III.A *supra*.

noninfringement position. I have been informed and understand from counsel that Dr. Van Ness has thus waived an interpretation on these terms that differs from their plain and ordinary meaning. If the Court were nevertheless to permit Dr. Van Ness to offer additional opinions on claim construction for those terms Dr. Van Ness has not purported to construe, I reserve the right to respond and amend or supplement my opinions.

267. With regard to Dr. Van Ness’s construction of “amplifying,” I disagree with Dr. Van Ness’s construction because it contradicts the plain language of the claim as explained *supra*.³¹⁹ Moreover, it is premised on a re-writing of the claim to inject the term “directly” where it does not exist. Claim 1 of the ’454 Patent recites “comprising,” which has an open-ended plain meaning and allows for other unrecited steps or elements to be present during the infringing method. Because Dr. Van Ness’s interpretation of these terms is incorrect, his opinions on noninfringement are likewise incorrect.

268. As I explained in my Opening Declaration, the Asserted Claims of the Natera Patents are composed of terms the plain and ordinary meaning of which would have been known to one of ordinary skill in the art.³²⁰ I therefore used the plain meaning of the terms for my infringement analysis.³²¹ It is my opinion that the accused RaDaR assay infringes the Asserted Claims of the Natera Patents and the presence of a universal PCR reaction after the targeted multiplex reaction does not alter my conclusion.

³¹⁹ See Section **Error! Reference source not found..**

³²⁰ See Metzker Op. Decl. ¶¶24, 54.

³²¹ *Id.* § VIII

(ii) *Dr. Van Ness's Reliance on "Amplifying"*

269. Dr. Van Ness opines that the accused RaDaR assay does not infringe because, in his view, it does not "directly" sequence the amplicons from the targeted multiplex reaction.³²² I disagree.

270. First, the claim language itself does not require "direct" sequencing of anything, including any amplified DNA product. That term does not appear anywhere in claim 1. The claim states "sequencing the amplicons to obtain sequence reads," without any reference to that sequencing being "direct." In my opinion, the limitation "sequencing the amplicons to obtain sequence reads" of claim 1 is met by the accused RaDaR assay by its sequencing step. While the amplified products of the targeted PCR reaction are further amplified to add sequencing-related sequences (adaptors and barcodes), the same sequence generated by the targeted multiplex reaction (50-150 bp comprising the target loci) is subjected to sequencing, and sequence reads for that sequence are obtained.

271. Second, as mentioned *supra*, this noninfringement opinion relies on a flawed interpretation of the claims, including the term "the amplicon." Under a plain and ordinary understanding, Claim 1 is an open-ended claim by virtue of its use of the term "comprising" in the preamble. I understand from counsel that "comprising" claims can be infringed even in the presence of unrecited steps or elements, so long as the other elements of the claims are met. Claim 1 recites "performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free

³²² See Van Ness Decl. at ¶¶79-80, 87; Van Ness Tr. at 235:5-236:25.

DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases” and is open-ended by the use of “comprising” recited earlier in Claim 1 of the ’454 Patent. One of ordinary skill in the art would have understood from the claim language itself that the sole restriction on “amplicons” is based on a length of 50-150 base pairs.

272. One of ordinary skill in the art would have readily understood that the “comprising” language allows for subsequent amplification(s) following targeted multiplex PCR and prior to sequencing. A subsequent amplification may be, for example, a universal PCR reaction to amplify those tags added to the ends of DNA and/or to attach additional sequencing adaptors for downstream sequencing. Subsequent amplification may also be a universal PCR to attach sequencing adaptors together with sample barcodes.

273. Third, dependent claim 11 of the ’454 Patent confirms that subsequent amplification(s) following targeted multiplex PCR and prior to sequencing are not excluded from Claim 1 of the ’454 Patent, as Dr. Van Ness argues. Claim 11 of the ’454 Patent recites “performing barcoding PCR prior to sequencing.”³²³ This barcoding PCR is a separate, intermediate PCR reaction from the targeted multiplex PCR and serves to add so-called “barcode” sequences so that different populations of DNA can be identified after sequencing. I am informed by counsel that Claim 1 of the ’454 Patent, as the independent claim from which Claim 11 depends, is presumed to be broader and to encompass the scope

³²³ See ’454 Patent at 172:1-3.

of Claim 11. Accordingly, Claim 1 of the '454 Patent permits, but does not require, additional PCR reactions for sequencing.

274. It is my opinion that Claim 1 of the '454 Patent encompasses a possible universal PCR step to add sequencing adaptors (or other sequencing-related sequences like barcodes) following targeted multiplex PCR in a single reaction volume, which one of ordinary skill in the art would have understood to be necessary for sequencing on certain instruments, including routinely and commonly-used Illumina sequencers which are mentioned repeatedly in the '454 Patent specification.³²⁴

275. For all these reasons, Dr. Van Ness's opinion is incorrect to the extent it excludes a step to add sequencing adaptors for sequencing (e.g., by universal PCR), conflicting with the plain and ordinary meaning of the claim language. This is made clear by his reading of the claim language that appears to inject the term "directly" where it does not exist.

(iii) Dr. Van Ness's Opinions on Natera's '008 Patent

276. In support of his non-infringement opinion, Dr. Van Ness also purports to rely on a separate, unasserted Natera patent—namely, the '008 Patent.³²⁵ I disagree that Natera's statements regarding the '008 Patent, which is not at issue in this case and claims different inventions, have any bearing on how the '454 Patent claims should be interpreted.

³²⁴ See, e.g., Meyer (2010).

³²⁵ See Van Ness Decl. at ¶¶79-80, 87.

277. First, the Van Ness Declaration refers to the '008 Patent as being “directly related to the '454 Patent” but fails to acknowledge that the two patents are part of separate lineages descending from the '703 Patent Appl.³²⁶ The '454 Patent claims priority to and is a great-granddaughter of the '703 Appl.³²⁷ The '703 Appl. also has *a separate lineage* of descendants, including a daughter '008 Patent.³²⁸

278. Second, while the '454 and '008 Patents share the same specification, their claims differ in several material ways, as shown *infra*:

US 11,530,454	US 11,486,008
<p>A method for preparing a plasma sample of a subject having cancer or suspected of having cancer useful for detecting one or more single nucleotide variant (SNV) mutations in the plasma sample, the method comprising:</p> <p>performing whole exome sequencing or whole genome sequencing on a tumor sample of the subject to identify a plurality of tumor-specific SNV mutations;</p> <p>performing targeted multiplex amplification to amplify 10 to 500 target loci each encompassing a different tumor-specific SNV mutation from cell-free DNA isolated from a plasma sample of the subject or DNA derived therefrom to obtain amplicons having a length of 50-150 bases, wherein the target loci are amplified together in the same reaction volume; and</p>	<p>A method for detecting one or more mutations or genetic variations in a biological sample of a subject having cancer or suspected of having cancer, the method comprising:</p> <p>identifying a plurality of tumor-specific mutations or genetic variations in a cancer sample of the subject by sequencing, wherein the mutations or genetic variations comprise one or more single nucleotide variant (SNV) mutations;</p> <p>collecting a biological sample from the subject, and isolating cell-free DNA from the biological sample;</p> <p>performing multiplex targeted amplification to amplify at least 100 target loci corresponding to the tumor-specific mutations or genetic variations from the isolated cell-free DNA to obtain amplicons, wherein the</p>

³²⁶ See Babiarz *et al.*, *Detecting mutations and ploidy in chromosomal segments*, U.S. Patent Application 14/692,703 (2015) (the “'703 Patent Appl.”).

³²⁷ See '454 Patent at cover page.

³²⁸ See Dkt. 108-1 ('008 Patent) at cover page.

<p>sequencing the amplicons to obtain sequence reads, and detecting one or more of the tumor-specific SNV mutations present in the cell-free DNA from the sequence reads,</p> <p>wherein the sequencing has a depth of read of at least 50,000 per target locus.</p>	<p>target loci are amplified together in the same reaction volume and not in separate reaction volumes;</p> <p>performing high-throughput sequencing to sequence the amplicons obtained in the multiplex targeted amplification reaction to obtain sequence reads; and</p> <p>detecting one or more of the mutations or genetic variations present in the cell-free DNA from the sequence reads, wherein an SNV mutation that is present in less than or equal to 0.015% of cell-free DNA molecules having the SNV locus in the biological sample is detected.</p>
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279. Notably, Claim 1 of the '454 Patent recites “sequencing the amplicons to obtain sequence reads” whereas Claim 1 of the '008 Patent refers to “performing high-throughput sequencing to sequence the amplicons obtained in the multiplex targeted amplification reaction to obtain sequence reads.” The underlined language is not present in any claim of the '454 Patent, evincing a difference in scope. The scope of Claim 1 of the '008 Patent is narrower than Claim 1 of the '454 Patent since Claim 1 of the '008 Patent requires that sequencing is performed on the amplicons directly resulting from multiplex targeted amplification reaction. Moreover, to the extent that Dr. Van Ness contends the dependent claims of the '008 Patent essentially cover the same claim scope as that of Claim 1 of the '454 Patent,³²⁹ I disagree. Because independent Claim 1 of the '008 Patent has a scope that is narrower compared to Claim 1 of the '454 Patent, the addition of any

³²⁹ See Van Ness Tr. at 212:24-215:4.

dependent claims will only further narrow the scope of claims in the '008 Patent, not broaden their scope to create overlap with that of the '454 Patent claims.

280. Third, Dr. Van Ness does not accurately describe Natera's back and forth with the patent examiner, which was based on a combination of prior art (Forsheaw (2012) and Takano (2005)) that was not raised against the '454 Patent (Forsheaw (2012) and Benesova (2013)).³³⁰ Dr. Van Ness asserts that Natera's assertions "require that any amplicons generated by performing a targeted multiplex amplification must be *directly* sequenced without any intervening step."³³¹ But this is not what Natera told the Examiner. Natera never referred to "direct" sequencing as Dr. Van Ness suggests. In distinguishing Forsheaw (2012), Natera referred specifically to the presence of "a subsequent and separate *targeted* amplification"³³²—not a *untargeted*, universal amplification to, for example, add sequencing adaptors and/or barcodes.

281. Fourth, Dr. Van Ness points to statements by Natera employee Dr. Bernard Zimmermann, an inventor on both the '454 and '008 Patents³³³, but again fails to note that these are statements about the '**008 Patent** claimed inventions, not the '454 Patent. The

³³⁰ Compare Dkt. 118-12 with Dkt. 116-5; compare Dkt. 118-10 with 116-9; see also Takano *et al.*, *Epidermal growth factor receptor gene mutations and increase copy numbers predict gefitinib sensitivity in patients with recurrent non-small-cell lung cancer*, JOURNAL OF CLINICAL ONCOLOGY 28:6829-6837 (2005) ("Takano (2005)"); Benesova *et al.*, *Mutation-based detection and monitoring of cell-free tumor DNA in peripheral blood of cancer patients*, Analytical Biochemistry 433:227-234 (2013) ("Benesova (2013)").

³³¹ See Van Ness Decl. at ¶ 80 (emphasis in original).

³³² See Dkt. 116-9 at 4 (emphasis added).

³³³ See Van Ness Decl. at ¶ 80.

CONFIDENTIAL MATERIAL OMITTED

'454 and '008 Patent inventions are separate and distinct, and the '008 Patent claims are in response to rejections not made against the '454 Patent claims. Dr. Zimmermann also did not say anything about universal PCR to add sequencing adaptors. In my opinion, Dr. Zimmermann's statements do not support Dr. Van Ness's opinions about the '454 Patent claims.

[REDACTED]

³³⁴ See '454 Patent at 171:40-41.

³³⁵ See Dkt. 107 at 6.

³³⁶ See Van Ness Tr. at 10:17-24.

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³³⁷ See Dkt. No. 112, *Declaration of Vishal Sikri in Support of Neogenomics Laboratories, Inc.’s Opposition to Natera, Inc.’s Motion for Preliminary Injunction*, dated October 18, 2023 (“Sikri Decl.”).

³³⁸ *Id.* at ¶ 49.

339 *Id.*

³⁴⁰ *Id.* at ¶¶ 50-51.

³⁴¹ See Dkt. 139-7 (Sikri Tr.) at 74:17-21.

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³⁴² *Id.* at 75:3-9.

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(v) *RaDaR infringes under the Doctrine of Equivalents*

288. As discussed *supra*, the accused RaDaR assay literally infringes the Asserted Claims of the '454 Patent. However, even if Dr. Van Ness's interpretations of certain claim terms were adopted by the Court, it is my opinion that RaDaR would still infringe the Asserted Claims of the '454 Patent under the doctrine of equivalents.

289. I understand from counsel that one way to evaluate infringement under the Doctrine of Equivalents is to determine whether an accused method performs substantially the same function in substantially the same way to achieve substantially the same result as the claimed method. Here, the incorporation of a routine and conventional universal PCR step after targeted multiplex amplification in a single reaction volume does not alter the functionality or result of the claimed method (*i.e.*, detecting one or more of the tumor-specific SNV mutations). It is also my opinion that the incorporation of a universal PCR

³⁴³ See Ex. 12 (NeoGenomics' Medicare Announcement dated July 27, 2023).

step after targeted multiplex amplification in a single reaction volume does not substantially alter the way the claimed method is performed.

290. For example, one of ordinary skill in the art would have understood that sequencing amplicons could require universal amplification to add sequencing adaptors (*e.g.*, sequencing adaptors are required for sequencing on an Illumina sequencer, which the '454 Patent teaches can be used for the sequencing step). I disagree with Dr. Van Ness's contention that this universal PCR step will be so indiscriminate such that "the relative amounts of mutations present in the original sample will be lost."³⁴⁴ Universal PCR generally uses common primer sequences, but will preserve the relative quantities of mutations present. One of ordinary skill in the art would have recognized that the relative quantities of mutations present can be preserved in a subsequent PCR by controlling the number of cycles performed. If the number of cycles remains within the exponential amplification phase, the relative abundance of mutations will not be diluted. Additionally, Dr. Van Ness fails to describe that in RaDaR, only the amplicons having the CS1/CS2 common tail adaptor sequences (first introduced through tagging) will be amplified in a universal PCR reaction since the universal PCR primers will hybridize to the common tail sequences. Therefore, universal PCR will amplify the final products from the targeted multiplex PCR step.

³⁴⁴ See Van Ness Decl. at ¶ 87.

291. I understand that Dr. Van Ness further contends that an additional PCR would necessarily introduce further polymerase error that would have materially impacted the results.³⁴⁵ I disagree. One of ordinary skill in the art would have readily recognized that an additional PCR could be performed using a proofreading polymerase (as opposed to *Taq* polymerase), such that there is no substantial increase in error.

292. I further understand that Dr. Van Ness contends that Natera may not assert a Doctrine of Equivalents theory for “sequencing the amplicons” based on an alleged disclaimer during prosecution of the ’008 Patent. I disagree that there has been any statement in the ’454 Patent prosecution that could be a disclaimer. Dr. Van Ness does not identify one.

293. To the extent that the Court permits Dr. Van Ness to supplement his noninfringement position regarding the ’454 Patent, I reserve the right to respond and supplement or amend my opinions.

B. ’035 Patent

294. As explained *infra*, I disagree with Dr. Van Ness’s contention that the accused RaDaR assay does not infringe the Asserted Claims of the ’035 patent.

295. Contrary to Dr. Van Ness’s assertions,³⁴⁶ RaDaR performs targeted amplification of *already* tagged DNA as part of its 15-cycle PCR. As I explained during

³⁴⁵ *Id.*

³⁴⁶ *Id.* at ¶¶ 89-104.

my deposition, the first cycle of the pre-amplification step disclosed by Forsheew (2012) tags cell-free DNA with universal tail adaptors CS1 and CS2.

296. Dr. Van Ness contends that tagging “cannot be satisfied by the first step of PCR because tagged products with both first and second universal tail adaptors are only generated in cycles after a first PCR cycle. During the first cycle a first tail adaptor is introduced. It is only after the first PCR cycle, at which point a second primer with a second tail adapter hybridizes to a copy of the original molecule, that products with the second tail adaptor are generated.”³⁴⁷ I disagree with Dr. Van Ness’s opinion and understanding of the foundations of the principles of PCR.

297. As explained *supra*,³⁴⁸ a single cycle of PCR can introduce **two tags** (one on each strand of the amplified DNA product). The subsequent cycles of PCR amplify the target region of interest, thereby performing targeted amplification of **already tagged** DNA. In mapping this scientific theory to the accused RaDaR assay, the first cycle of RaDaR’s preamplification PCR step introduces **two tags** (one on each strand of the amplified DNA product), which I have explained in my Opening Declaration.³⁴⁹ The subsequent cycles of PCR amplify the target region of interest, thereby performing targeted amplification of already tagged DNA.³⁵⁰ In my opinion, Dr. Van Ness’s theory that tagging requires two

³⁴⁷ *Id.* at ¶101.

³⁴⁸ *See* Section V.B(ii).

³⁴⁹ *See* Metzker Op. Decl. at ¶¶ 87-101.

³⁵⁰ *See id.*

cycles of PCR is inconsistent with the fundamental principles of PCR. Regardless, Dr. Van Ness's assessment does not change my opinion because even if Dr. Van Ness were correct about the two tags being added over two PCR cycles (not one), tagged products are still being subsequently amplified afterwards in every remaining PCR cycle.

298. Support for my opinion further derives from Claim 12, which recites: "the one or more universal tail adaptors comprise a first universal tail adaptor and a second universal tail adaptor." Therefore, even under the Van Ness Declaration's view of the second tag being added to an amplicon and not cell-free DNA, Dr. Van Ness does not deny that the first tagging step adds "one or more adaptors."³⁵¹

299. I also disagree with Dr. Van Ness's reliance on a dependent claim (Claim 13)³⁵² to attempt to narrow the scope of independent Claim 1 of the '035 Patent, which contradicts the plain meaning of Claim 1 as described *supra*.³⁵³

300. Therefore, it is my opinion that the first cycle of RaDaR's preamplification step satisfies the "tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged products" limitation of Claim 1 of the '035 Patent. Additionally, the subsequent cycles of RaDaR's preamplification step satisfy the "amplifying the tagged products one or more times." For this reason, it is my opinion that the accused RaDaR assays infringes the Asserted Claims of the '035 Patent.

³⁵¹ See Van Ness Tr. at 228:17-232:25.

³⁵² See Van Ness Decl. at ¶¶ 100-101.

³⁵³ See Section **Error! Reference source not found..**

(ii) *RaDaR infringes under the Doctrine of Equivalents*

301. As discussed *supra*, the accused RaDaR assay literally infringes the Asserted Claims of the '035 Patent. However, even if Dr. Van Ness's interpretations of certain claim terms were adopted by the Court, it is my opinion that RaDaR would still infringe under the Doctrine of Equivalents.

302. I understand that one way to evaluate infringement under the Doctrine of Equivalents is to determine whether an accused method performs substantially the same function in substantially the same way to achieve substantially the same result as the claimed method. Here, the incorporation of a universal PCR step in the accused RaDaR assay after targeted multiplex amplification in a single reaction volume does not substantially alter the functionality, the way, or result of the claimed method because the claimed method already includes "wherein one of the amplifying steps introduces a barcode and one or more sequencing tags," which one of ordinary skill in the art would have understood may be done through universal PCR.

303. For example, universal PCR generally uses common primer sequences, but will preserve the relative quantities of mutations present. One of ordinary skill in the art would have recognized that the relative quantities of mutations present can be preserved in a subsequent PCR by controlling the number of cycles performed. If the number of cycles remains within the exponential amplification phase, the relative abundance of mutations will not be diluted. Additionally, Dr. Van Ness fails to describe that in RaDaR, only the amplicons having the CS1/CS2 common tail adaptor sequences (first introduced through

tagging) will be amplified in a universal PCR reaction since the universal PCR primers will hybridize to the common tail sequences. Therefore, universal PCR will amplify the final products from the targeted multiplex PCR step.

304. I understand that Dr. Van Ness further would necessarily introduce further polymerase error that would have materially impacted the results. I disagree. One or ordinary skill in the art would have readily recognized that an additional PCR could be performed using a proofreading polymerase (as opposed to *Taq* polymerase), such that there is no substantial increase in error.

305. To the extent that the Court permits Dr. Van Ness to supplement his noninfringement position regarding the '035 Patent, I reserve the right to respond and supplement or amend my opinions.

X. SIGNATERA PRACTICES THE NATERA PATENT CLAIMS

306. I previously described how Signatera practices Claim 1 of each of the Natera Patents in my Opening Declaration.³⁵⁴ I understand that Dr. Van Ness opines that Signatera does not practice Claim 1 of the '454 Patent nor Claim 1 of the '035 Patent. Dr. Van Ness testified during his deposition, however, that he did not review any of Natera's technical documents that have been produced to date in reaching this alleged conclusion.³⁵⁵ I disagree with Dr. Van Ness's opinions as described *infra*.

³⁵⁴ See Metzker Op. Decl. at § IX.

³⁵⁵ See Van Ness Tr. at 246:4-20.

A. '454 Patent

307. With respect to the '454 Patent, Dr. Van Ness opines that Signatera does not practice the limitation of “amplicons having a length of 50-150 bases.”³⁵⁶ I disagree with Dr. Van Ness. One of Natera’s technical documents states the following:

The prioritized list of variants is used to design PCR amplicons based on optimized design parameters, ensuring uniqueness of the amplicon sequences in the human genome and efficiency of the amplicons. *Each amplicon can be designed to amplify sequences of up to 70 base pair length* and is designed to contains one base pair that differs between the tumor and somatic genotypes.³⁵⁷

308. Therefore, it is my opinion that Signatera does practice the aforementioned limitation of Claim 1 of the '454 Patent.

B. '035 Patent

309. With respect to the '035 Patent, the Declaration of Dr. Van Ness opines that Signatera does not practice the limitation of “amplifying the tagged products one or more times to generate final amplification products . . . wherein one of the amplifying steps introduces a barcode and one or more sequencing tags.”³⁵⁸ I disagree with Dr. Van Ness’s opinions.

310. In my Opening Declaration, I describe how Signatera practices “tagging isolated cell free DNA with one or more universal tail adaptors to generate tagged

³⁵⁶ See Van Ness Decl. at ¶¶ 428-438.

³⁵⁷ See Van Ness Tr. at Ex. 30, NAT-NEO-00625926-00625931 (“Device description for Signatera”) at NAT-NEO-00625928 (emphasis added).

³⁵⁸ See Van Ness Decl. at ¶¶ 439-442.

products.”³⁵⁹ I relied upon Coombes (2019),³⁶⁰ Christensen (2019),³⁶¹ and Kotani (2023)³⁶² as support.³⁶³ Dr. Van Ness does not refute my opinion that Signatera practices this step. My opinion is further supported by Natera’s internal technical documentation, which states, “[t]he cfDNA is end-repaired, A-tailed, and ligated with custom adapters, as previously described in Abbosh *et al.* 2017. The purified ligation product is amplified.”³⁶⁴

311. Dr. Van Ness argues that Signatera does not practice the limitation of “amplifying the tagged products one or more times to generate final amplification products. . . wherein one of the amplifying steps introduces a barcode and one or more sequencing tags”³⁶⁵ even though the references on which I rely (*e.g.*, Coombes (2019) and Christensen (2019)) show how Signatera practices this limitation, as explained in my Opening Declaration.³⁶⁶ My opinion is further supported by Natera’s internal technical

³⁵⁹ See Metzker Op. Decl. at ¶¶ 133-134.

³⁶⁰ See Dkt. 17-19, Coombes *et al.*, *Personalized detection of circulating tumor DNA antedates breast cancer metastatic recurrence*, CLINICAL CANCER RESEARCH 25:4255–4263 (2019) (“Coombes (2019)”).

³⁶¹ See Dkt. 17-21, Christensen *et al.*, *Early detection of metastatic relapse and monitoring of therapeutic efficacy by ultra-deep sequencing of plasma cell-free DNA in patients with urothelial bladder carcinoma*, JOURNAL OF CLINICAL ONCOLOGY 37:1547-1557 (2019) (“Christensen (2019)”);

³⁶² See Dkt. 17-22, Kotani *et al.*, *Molecular residual disease and efficacy of adjuvant chemotherapy in patients with colorectal cancer*, NATURE MEDICINE 29:127-134 (2023) (“Kotani (2023)”).

³⁶³ See Metzker Op. Decl. at ¶¶ 133-134.

³⁶⁴ See Device description for Signatera at NAT-NEO-00625929.

³⁶⁵ See Van Ness Decl. at ¶¶ 439-442.

³⁶⁶ See Metzker Op. Decl. at ¶ 135.

documentation.³⁶⁷ Dr. Van Ness does not address the disclosure of Coombes (2019) and Christensen (2019) nor any of Natera's technical documents in forming his opinions. Dr. Van Ness's opinions regarding Signatera are thus flawed.

312. Therefore, it is my opinion that Signatera does practice the aforementioned limitation of Claim 1 of the '035 Patent.

³⁶⁷ See Device description for Signatera at NAT-NEO-00625930.

I confirm that the contents of this Reply Declaration are true to the best of my knowledge and belief insofar as it states facts and that it contains my honest opinions on the matters upon which I have been asked to give them.

I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Dated: November 6, 2023

Michael Metzker, Ph.D.

I confirm that the contents of this Declaration are true to the best of my knowledge and belief insofar as it states facts and that it contains my honest opinions on the matters upon which I have been asked to give them.

I declare under penalty of perjury under the laws of the United States of America that the foregoing is true and correct.

Dated: November 6, 2023

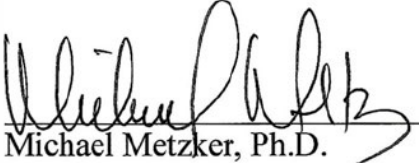

Michael Metzker, Ph.D.

EXHIBIT 2

Cell-free DNA (cfDNA): Clinical Significance and Utility in Cancer Shaped By Emerging Technologies

Stanislav Volik¹, Miguel Alcaide², Ryan D. Morin^{2,3}, and Colin Collins^{1,4}

Abstract

Precision oncology is predicated upon the ability to detect specific actionable genomic alterations and to monitor their adaptive evolution during treatment to counter resistance. Because of spatial and temporal heterogeneity and comorbidities associated with obtaining tumor tissues, especially in the case of metastatic disease, traditional methods for tumor sampling are impractical for this application. Known to be present in the blood of cancer patients for decades, cell-free DNA (cfDNA) is beginning to inform on tumor genetics, tumor burden, and mechanisms of progression and drug resistance. This substrate is amenable for inexpensive noninvasive testing and thus presents a viable approach to serial sampling for screening and monitoring tumor

progression. The fragmentation, low yield, and variable admixture of normal DNA present formidable technical challenges for realization of this potential. This review summarizes the history of cfDNA discovery, its biological properties, and explores emerging technologies for clinically relevant sequence-based analysis of cfDNA in cancer patients. Molecular barcoding (or Unique Molecular Identifier, UMI)-based methods currently appear to offer an optimal balance between sensitivity, flexibility, and cost and constitute a promising approach for clinically relevant assays for near real-time monitoring of treatment-induced mutational adaptations to guide evidence-based precision oncology. *Mol Cancer Res*; 14(10); 898–908. ©2016 AACR.

Early Discovery and Applications of cfDNA

The presence of cell-free DNA (cfDNA) in blood plasma was discovered in 1948 by Mandel and Metais (1). Seventeen years later, in 1965, Bendich and colleagues hypothesized, that cancer-derived cfDNA could be involved in metastasis (2). However, it took another year to discover the first link to disease. In 1966, Tan and colleagues observed high levels of circulating cell-free DNA (cfDNA) in the blood of systemic lupus erythematosus patients (3). Eleven years later, in 1977, Leon and colleagues used radio-immunochemistry to demonstrate that for at least half of cancer patients the level of cfDNA in their blood was significantly higher than in normal control subjects (4). The authors noted that patients with metastatic cancer had significantly higher cfDNA levels in blood. Because of technological limitations, it took

another 12 years for the first experimental evidence to support that cfDNA in cancer patients does indeed contain tumor DNA based on temperature stability measurements (5).

The technological progress of the 1990s fuelled by the Human Genome Project allowed more direct demonstration for a tumor origin of at least some cancer patient cfDNA. In 1994, two groups reported the presence of tumor-specific mutations in cfDNA (6, 7). Both groups used mutation-specific primers to facilitate PCR amplification of tumor-specific (*N-RAS*) mutations in the plasma samples of patients with pancreatic adenocarcinoma and acute myelogenous leukemia (AML), respectively. This approach to detection of specific *a priori* known mutations in cfDNA was to become the preferred method of cfDNA studies until the advent of massively parallel sequencing (MPS). Circulating tumor DNA (ctDNA) is typically so diluted by normal DNA that existing sequencing methods (e.g., Sanger sequencing) were not sufficiently sensitive to detect mutant DNA molecules. As a result, mutation-specific PCR was the only available technology that could provide sufficient specificity for detection of the weak tumor signal. It was recognized in these pioneering studies that the detection of tumor DNA in circulation offers exciting implications for clinical translation for "diagnosis, determining response to treatment, and predicting prognosis" (7). Not surprisingly, soon after this initial breakthrough essentially all other types of tumor-specific DNA changes were discovered in cfDNA, such as changes in the status of microsatellite markers including loss of heterozygosity (LOH; refs. 8, 9); gene amplifications (10, 11); the presence of the oncogenic viral DNA (12–15); and hypermethylation of the promoter regions of tumor suppressor genes (16–18). While these early observations highlighted many possibilities for using ctDNA as a noninvasive approach to analyze tumor genomes, sufficiently sensitive and specific laboratory techniques to fully leverage this potential were not yet developed.

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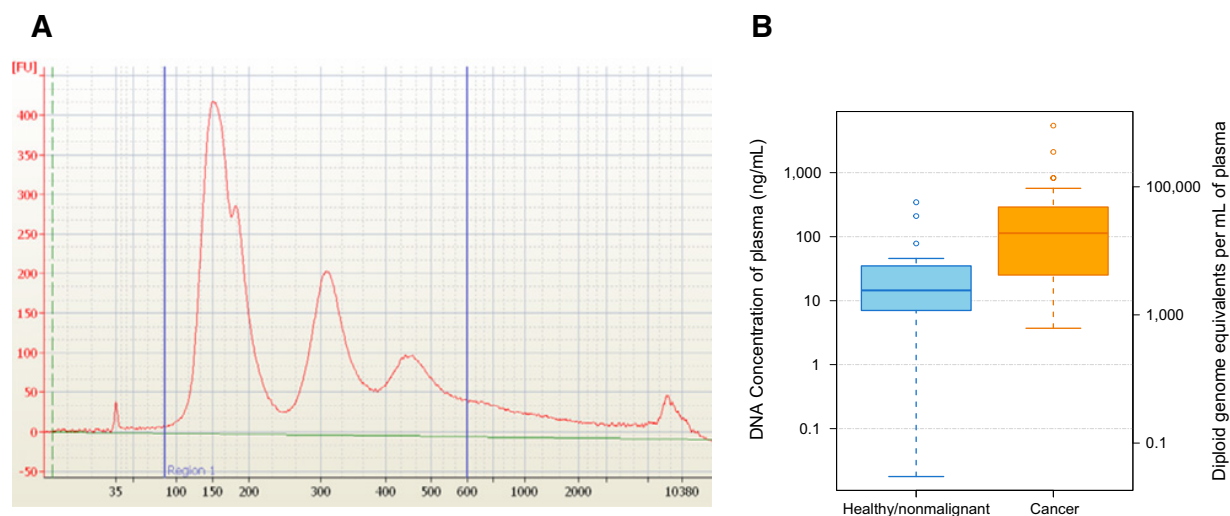
Note: Supplementary data for this article are available at Molecular Cancer Research Online (<http://mcr.aacrjournals.org/>).

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doi: 10.1158/1541-7786.MCR-16-0044

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**Figure 1.**

A, a Bioanalyzer 2100 (Agilent Technologies) profile showing the length distribution of cfDNA isolated from a high-risk prostate cancer patient reported in ref. 31. The x-axis shows the size of DNA fragments (in base pairs); the y-axis, fluorescent intensity, proportional to DNA concentration. Note the unusual trimodal distribution of DNA sizes (150, 300, and 450 bp) most likely indicating a very high tumor burden. **B**, cfDNA concentration in blood of healthy subjects/nonmalignant patients (blue box) and in cancer patients (orange box) based on ref. 30. The left y-axis shows the cfDNA concentration on a logarithmic scale (in ng/mL of blood) and the right shows the number of diploid genome equivalents per mL of blood (assuming 6 pg of DNA per diploid genome).

The "chimeric" nature of cfDNA, the presence of both normal and tumor DNA in blood plasma, enabled development of applications in other fields. However, these are outside of the scope of the current review and will only be briefly mentioned here. Arguably the most successful application of cfDNA studies was the discovery of the high admixture of fetal-derived cfDNA in mother's blood stream by Lo and colleagues (19). Later the same group demonstrated that in 70% of women bearing male fetuses, fetal Y-chromosome sequences could be detected in just 10 μ L of blood plasma (20). This discovery opened up a new avenue for development of fetal cfDNA-based prenatal genetic testing. For example, in one recent large study involving 1,914 women across 21 U.S. centres it was shown that cfDNA-based prenatal testing for fetal aneuploidy has a significantly lower false positive rate for detection of trisomies 21 and 18 compared with standard procedures (10 times lower for trisomy 21 and 3 times lower for trisomy 18) and significantly higher negative and positive predictive values (ref. 21; for detailed review of prenatal diagnostics application of cfDNA, see ref. 22). Another interesting application of cfDNA-based detection of "foreign" DNA is monitoring the status of solid organ transplants. As DNA is mostly released into the blood as a result of cell death, the level of the donor's DNA in recipients blood can be used as a marker of rejection (for detailed review of this cfDNA application, see ref. 23).

Later studies linked cfDNA levels to outcomes in severe injury such as blunt trauma and burns (reviewed in ref. 24). cfDNA levels correlated with the length of hospital stay, burn surface area, the number of operations needed for scalds (though not for the flash/flame burns). Plasma cfDNA levels also correlated with the need for patient ventilation in intensive care units (ICU). High (above 127 ng/mL) concentrations of cfDNA in blood were found to be a predictor of death for ICU patients (with sensitivity of 92% and specificity of 82%). In line with these findings, cfDNA levels in blood turned out to be higher and have certain predictive value for

sepsis and septic shock, aseptic inflammation, myocardial infarction, stroke including patients with negative neuroimaging results, where cfDNA concentrations seem to predict poststroke morbidity and mortality in patients with negative neuroimaging, and sickle cell disease. In short, cfDNA concentration is elevated in conditions that involve increased rates of cell death and necrosis.

Properties of cfDNA

Multiple properties of cfDNA suggest cell death as its major origin. Importantly, cfDNA is double stranded and highly fragmented, with most molecules being approximately 150 bp in length (Fig. 1A). This matches the length of DNA occupied by a nucleosome, the primary unit for spatial organization of DNA in the nucleus (25). Moreover, the other fragment length peaks correspond well with linear progression of nucleosome units (two units for 300-bp band, three units for 450-bp band; Fig. 1A). Interestingly, there is still controversy on whether the higher or lower integrity of cfDNA is associated with cancer. In 2003, Wang and colleagues reported that the comparisons of the relative amounts of 100- and 400-bp PCR products of the β -actin gene demonstrated increased cfDNA integrity in 61 patients with breast and gynecologic cancers compared with 65 non-neoplastic patients (26). This observation is supported by the studies in numerous cancer types summarized in ref. 27. However, there are also conflicting reports, including by Madhavan and colleagues (27), who determined that decreased cfDNA integrity (defined as the ratio of concentrations of long, \sim 260-bp Alu and LINE fragments to short, \sim 100 bp, fragments determined by qPCR) correlates with worse outcome. The authors noted that the decreased cfDNA integrity would imply higher apoptotic rates, and that increased apoptosis correlates with higher tumor proliferation. This in turn would imply that the apoptotic, not necrotic cells, are the main source of cfDNA at least in cancer

patients (28). One corollary of this supposition is that tumors with higher proliferation may naturally yield higher proportion of cfDNA in line with well-established link between cell proliferation and apoptosis rates (for review, see ref. 29). In any case, all of the studies and our experience agree, that as much as 90% of the total cfDNA is contained in low molecular weight band (~150–180 bp).

The amount of cfDNA in cancer patients varies widely. A good summary can be found in 2008 Fleischhacker and Schmidt review (30) who assembled the results of 34 studies involving healthy subjects and patients with malignant and nonmalignant disease (summarized in Fig. 1B). While a trend toward the DNA concentration in the blood of cancer patients being much higher than in the blood of healthy controls and nonmalignant patients is clear, the cfDNA concentration varies considerably and is below 100 ng/mL for the majority of reported cancer patients. This is in line with our own data—in a group of 62 castrate-resistant metastatic prostate cancer (mCRPC) patients the mean cfDNA concentration was 53 ng/mL of blood (31). Another way to look at these numbers is to calculate how many genome equivalents can be identified in blood. Assuming 6 pg of DNA per diploid human genome, the majority of cancer patients have below 17,000 genome equivalents per mL of blood. Patients from our recent study (31) had on average ~9,000 genome equivalents per mL of blood.

Not unexpectedly, in addition to varying absolute levels of cfDNA, the fraction of DNA molecules in the circulation of cancer patients that can be recognized as being derived from tumor cells also varies. In 2001, Jahr and colleagues published one of the first attempts to estimate the proportion of circulating tumor DNA (ctDNA) to total cfDNA (32). The ctDNA/cfDNA ratio was determined by quantifying the amount of hypermethylated *CDKN2A* promoter that the authors assumed to be tumor-specific. Hypermethylation of the *CDKN2A* promoter was detected in 11 of 25 specimens, "in line with previous studies," and in all six cases where both tumor tissue and cfDNA was available, results of methylation-specific PCR were concordant. The proportion of tumor-specific hypermethylated *CDKN2A* sequences ranged from <10% to >90% of the total cfDNA. Four years later, Diehl and colleagues published a study (33) in which they reported that the tumor content in cfDNA of 33 colorectal cancer patients ranged from 0.01% to 1.7%. Interestingly, they also reported, that the percentage of mutant molecules of the APC gene increased 5- to 20-fold when the fragment size used for PCR decreased from 1,296 to 100 bp. Numerous studies have shown similar results. For example, amplicon sequencing of the *PIK3CA* and *TP53* genes and digital PCR of the identified structural variations and point mutations allowed Dawson and colleagues to determine that in metastatic breast cancer the ctDNA defined as a fraction of the somatic mutant allele comprised a median of 4% of total cfDNA (interquartile range 1–14; ref. 34). This is in line with our study (31), where we identified mutations in exon 8 of the AR gene in cfDNA of metastatic castrate resistant prostate cancer patients at a frequency of 0.1%–23% (median 1.5%). Given the total cfDNA content estimated above we expect the most cancer patients to have less than 3,500 tumor genomes per mL of blood. In our cohort of mCRPC patients (31), the median yield of tumor DNA was approximately 135 genome equivalents per mL of blood.

Finally, an important characteristic of cfDNA is its rapid turnover. The first report in the kinetics of foreign DNA clearance from animal's blood dates back to 1963, when Tsumita and Iwanaga

used tritiated DNA injected into mice to show that 99% of the radioactivity is cleared from the bloodstream in 30 minutes (35). They have also reported the highest increase of radioactivity in kidneys, followed by liver and spleen, suggesting the importance of renal clearing of cfDNA. Later, in 1999, Lo and colleagues measured the half-life of the fetal cfDNA in mother's blood post-partum using real-time quantitative PCR of the *SRY* gene (36). They reported that the half-life of the male fetal DNA in women post-partum was 16.3 minutes, no detectable male-derived cfDNA were found in the mother's blood 2 hours after birth—a result that is very close to the original 1963 observation. Other studies confirmed the very short half-life of cfDNA in blood stream. For example, Fatouros and colleagues have measured the kinetics of cfDNA concentrations in athletes following vigorous exercise and reported that cfDNA increased to 15-fold postexercise, stabilized at 13-fold for 30 minutes after exercise and normalized 30 minutes later (37). In short, there is no controversy on the kinetics of cfDNA clearance from blood stream; however, the mechanisms of its clearance have not been studied in detail.

Collection and Processing of Blood for cfDNA

As discussed above, a large variability in the total plasma cfDNA levels has been reported among patients. Some of this variability may be explained by biological differences between patients, whereas some relates to different sensitivities of the analytic technologies employed by groups and even sources of contaminating DNA. Therefore, it is important to keep in mind that not all samples should be considered equivalent and that there are preanalytic considerations one should make when prospectively collecting samples specifically for cfDNA analyses. Much of our understanding of the biology of cfDNA and optimal methods for its collection and extraction comes from the study of fetal cfDNA for prenatal screening. Owing to the ability to readily distinguish fetal from maternal DNA (particularly with a male fetus), rigorous experiments have identified factors that affect yield and stability of cfDNA as well as sources of contamination (i.e., from maternal cells). For example, in 2001, Chiu and colleagues reported that different methods of isolation of cfDNA from the blood of pregnant women (such as filtration through 0.22- μ m filters, centrifugation in Percoll gradients, and high-speed centrifugation) produced significantly different amounts of maternal but not fetal cfDNA (38). Not surprisingly, they have noted the importance of standardization at the level of blood collection, processing, and DNA extraction so that samples within individual studies remain comparable.

At about the same time, ruptured blood cells were identified as a main source of cfDNA contamination (i.e., ref. 39), which has largely shaped efforts on optimization of the cfDNA isolation protocols. Although there is no clear consensus on the best practices for sample handling, we refer the reader to a review covering preanalytic variables that affect cfDNA quality (40). One key observation reported therein is that while overall, serum may yield higher levels of cfDNA than plasma, the yield is more variable and the cfDNA quality may be severely impacted due to lysis of monocytes. Plasma is theoretically less likely to be contaminated with DNA from blood cells but, importantly, the time elapsed between blood collection and centrifugation can heavily influence this (41). Unfortunately, because the overall yield of DNA extracted from plasma or the amount of DNA

measured by qPCR is often used as a proxy for cfDNA, there is also conflicting evidence on the extent of contamination from the blood (42). As revisited later, contamination may be best assessed by determining the relative abundance of high molecular weight DNA fragments that are not consistent with the apoptotic signature characteristic of cfDNA. Methods to overcome such contamination or even accurately assess it remain under development. To better circumvent this issue, at the British Columbia Cancer Agency and Vancouver Prostate Centre, we have opted to collect blood in EDTA tubes and separate plasma by double centrifugation at 1,600 rpm within two hours after collection. It is important to note that heparin tubes are not typically compatible with ctDNA detection methods because in our experience the effect of heparin on polymerase activity can severely impact sensitivity. There are some options for analyzing cfDNA from heparin-containing tubes or tubes containing other PCR inhibitors including polymerases that are more robust to such inhibitors. However, in our opinion for prospective studies, these contaminants are best avoided (43). Understandably, so-called rapid processing of blood is not feasible in all settings (e.g., blood collection at sites lacking a centrifuge) and may not be cost-effective in smaller centers. Preservatives such as formaldehyde have also been proposed as a means to prevent cell lysis thereby obviating the necessity for rapid processing. However, owing to its potential to damage DNA, other preservatives may be preferable, although we are not aware of any data showing a higher level of noise in cfDNA exposed to formaldehyde-based preservatives. An alternative are the cell-free DNA BCT tubes from Streck. These are advertised to prevent lysis at ambient temperature for up to two weeks but we and others have noted that processing within a week or less is more appropriate to minimize contamination (44). In summary, procedures for collecting plasma for cfDNA analysis should be standardized within centers (and studies) and, depending on the available infrastructure, one may opt to use rapid processing or cell-stabilizing tubes to minimize the risk of contamination from cellular fractions.

Biological Role of cfDNA

Despite almost 70 years of history, the biological function of cfDNA has only been unambiguously established for immune response and blood coagulation (28) and its function in other conditions, if any, remains nebulous. Neutrophils in human blood release so called neutrophil extracellular traps (NET) as one of responses to bacterial infection. cfDNA is an important component of these NETs, that allows them to bind and trap microbial pathogens. The release of DNA from neutrophils is thought to occur via an alternative mechanism of cell death termed NETosis (see refs. 45 and 46 for details). Briefly, two mechanisms are being considered, with the first involving dissolution of the plasma and nuclear membranes that is followed by release of the chromatin into extracellular space. Unlike apoptosis, NETosis does not result in display of the phagocyte-activating signals, so the neutrophils that undergo NETosis do not get cleared from the blood stream by phagocytes. An alternative theory postulates the existence of a DNA/serine nuclease extrusion mechanism from intact neutrophils and that autophagy contributes to NETosis. In any case, NETs appear to require intact chromatin lattices. Importantly, cfDNA in NETs triggers blood coagulation, a process that clearly needs to be tightly controlled. This control is affected by the DNaseI in the blood stream. It has

also been suggested, that another important function of the DNaseI-based mechanism of clearing cfDNA from human blood is the prevention of autoimmunity against DNA (47). Involvement of cfDNA was suggested for other biological processes as well, ranging from tumor dissemination (2, 48–50) to aging (51), but experimental evidence supporting such hypotheses remains tenuous, and considerable additional research is needed to clarify the involvement of cfDNA in processes other than immunologic response.

Genomic Analysis of cfDNA

The presence of tumor-derived DNA in cfDNA implies the entire spectrum of tumor genome aberrations are present and can thus be detected. However, the low amount, high degradation, and high admixture of normal DNA in cfDNA pose major challenges for the development of sensitive and robust detection pipelines. The fact that most (if not all) tumors are characterized by multiple subclonal populations with only a subset of somatic mutations shared among all cells (for review, see ref. 52) further complicates the issue. Broadly, current approaches for detection of tumor aberrations in cfDNA can be divided into two categories: methods targeting specific changes and methods allowing detection of all possible aberrations in DNA (including targeted and whole exome/genome sequencing). The latter next-generation sequencing (NGS)-based options offer numerous potential benefits for observing clonal differences in tumor cell populations, an advantage that until very recently was offset by more limited sensitivity and specificity.

Assessing specific DNA changes

A priori knowledge of specific DNA aberrations (mainly mutations and short insertions/deletions) and recurrent "hot spot" mutations allow implementation of a variety of sophisticated PCR-based methods for their detection in the cfDNA of cancer patients. These approaches historically had low DNA requirements and low noise levels, and proved quite efficient for cancer types where few genomic changes are important for the patient stratification.

Historically, one of the first applications for PCR-based analysis of ctDNA was the detection of high-level amplification of oncogenes. One of the earliest examples was the detection of increased levels of *MYCN* sequences in circulation in patients with neuroblastoma. *MYCN* amplification is a major prognostic factor in localized neuroblastoma. In 2002, Combaret and colleagues demonstrated that PCR and qPCR detect the presence of *MYCN* in blood of patients with *MYCN* amplification, but not in the blood of patients without amplification or healthy controls (11). However, this approach was not very efficient for patients with stage I and II disease (53), probably due to low cfDNA content and insufficient sensitivity of the employed methodology, as *MYCN* locus is present in a diploid state in all healthy cells and thus should be present in all cfDNA samples.

However, in general, detection of copy number aberrations (CNA) in cfDNA using aCGH or approaches that target single/limited loci targeting such as digital PCR has limitations that make it less appealing for ctDNA quantification. After all, unlike other types of cancer-specific mutations (i.e., point mutations or breakpoint detection), the assay must be capable of reliably distinguishing a small change in DNA copy number in a high background of diploid genomes. One of the first approaches to solve this

problem was measuring the number of copies of a locus of interest relative to one or more reference loci assumed to be diploid in all cells. For example, a digital PCR assay to detect *ERBB2* amplifications compared the number of copies of this locus to another gene on the same chromosome and applied a threshold that could distinguish plasma of *ERBB2*-amplified patients to unamplified patients at sensitivity of 64% and specificity of 94% (54). In general, such approaches are expected to be confounded by variability in copy number across patients. The sensitivity of CNA detection is also expected to suffer for patients with lower ctDNA abundance levels. Sequencing-based methods that broadly survey the genome have shown to be useful for cfDNA copy number profiling (i.e., ref. 55; discussed more below) when employed over large genomic regions, as is the case in prenatal screening for chromosome trisomies. Copy number status evaluation for smaller targeted panels remains challenging, especially for approaches that do not include the routine use of matched normal DNA.

Still, PCR-based techniques are widely used for the detection of specific mutations, and can broadly be classified as either qualitative or quantitative in nature. Qualitative methods generally provide a yes/no readout for the presence of the target mutation and include amplification refractory mutation system (ARMS) PCR (56, 57); PNA clamping PCR (58) and ligation-based methods that use DNA ligase and wild-type and mutation-specific reporter probes to quantify mutant DNA (59). The major disadvantage of these approaches is, obviously, a lack of precise quantification of mutant DNA molecules. Digital methods include various implementations of digital PCR (dPCR) approaches (60, 61), usually in the form of emulsion PCR (62), including BEAMing [for Beads, Emulsions, Amplification, and Magnetics] technology (33, 63)]. This family of approaches incorporate a number of techniques used to improve specificity and sensitivity of mutation detection by PCR through separation of template molecules into individual reaction vessels with modern methods typically using either microfluidics (64), by separating sample and PCR reagents into droplets in an oil emulsion (65, 66) or by combining microfluidics and emulsion PCR to generate evenly sized droplets (67).

BEAMing is an interesting example, because it incorporates a number of techniques used to improve specificity and sensitivity of mutation detection by PCR. Briefly, this approach consists of the following steps: first, the cfDNA mixture is PCR amplified using primers that introduce sequence tags into the resulting amplicons. Then amplicons are combined with streptavidin-coated magnetic beads, coated with nested primers, and emulsified so that each drop in emulsion contains on average one bead and one DNA fragment and emulsion PCR is performed that results in clonal amplification of each template on the surface of the beads. The PCR emulsion is deemulsified and DNA-covered beads are magnetically purified and the DNA on beads is hybridized with oligos complementary to a sequence adjacent to the nucleotide of interest. Next, a single base extension is performed using fluorescently labeled bases that allow differential labeling of the wild-type and mutant alleles. Finally, fluorescently labeled beads are counted/purified on a flow cytometer (and optionally validated by Sanger sequencing).

BEAMing was the first method to allow quantitative sensitive interrogation of mutant cfDNA. In this study, the authors noted that the sensitivity of the detection of rare mutant fragments was mainly limited by two factors: the number of genome equivalents entering the assay (in other words DNA fragments

spanning a given mutation and by the fidelity of the DNA polymerases employed in the two PCR steps). These inputs ranged from 1,350 to 230,000 per mL of blood in cancer patients and 1,150 to 8,280 fragments per mL of blood for control subjects, very close to our estimates above. However, most importantly, the errors introduced in the first PCR rounds cannot be eliminated because they would result in beads with homogeneous nonreference fragments, indistinguishable from the bona fide homozygous mutations (33). Interestingly, for this particular study the polymerase error rate was relatively less important than the limitations imposed by the low available amount of input cfDNA because of the possibility to use high-fidelity proofreading DNA polymerases and scoring of only specific base changes. The authors empirically determined that for the assessed targets and used polymerases the error rate after 30 PCR cycles was approximately 2×10^{-5} . The necessity of detecting multiple nonreference reads (three in this case) for identification of a mutation would limit sensitivity to the detection of nonreference base in $>1/1,333$ molecules or approximately 7.5×10^{-4} if 4,000 total cfDNA fragments were assayed (33). To conclude, since its publication, BEAMing has become commercialized and remains a research staple of some groups. For example, a recent application to plasma from patients with colon cancer allowed detection of circulating *KRAS* mutations, which are known to be acquired in response to EGFR blockade (68). It has also been extended for detection of methylated fragments (69).

In conclusion, even an early dPCR assay for quantifying mutant *KRAS* DNA suggested a sensitivity as high as one molecule in a background of 200,000 (70). This study also introduced the concept of combining multiple assays for different mutant alleles in a single experiment using different dye dilutions for individual alleles. Commercial kits that facilitate the multiplex screening of hot spots in *KRAS* now exist. Clearly, methods targeting specific sets of changes, including so-called "actionable" alterations, are of particular interest in clinical settings. By observing the presence of such mutations in a ctDNA sample, one can consider this as a "liquid biopsy" that could inform the clinician on a suitable treatment course. Currently, there are few drugs whose indication is associated with the presence or absence of specific genomic changes (Rubio-Perz and colleagues have listed 57 FDA-approved agents targeting 51 driver genes as of 2015 (71)). Therefore, a relatively small panel can test a patients' cfDNA for a large proportion of clinically relevant mutations with high specificity and sensitivity, which would be highly dependent on the level of ctDNA in the patient. However, as the number of targeted genes grows (Rubio-Perez and colleagues have counted a total of 96 targetable cancer driver genes if all current clinical trials are included; ref. 71) such assays become ever more unwieldy. This problem is exacerbated by the fact that many relevant mutations in cancer are not sufficiently recurrent to facilitate broad coverage using dPCR-based assays. Furthermore, copy number information can also be of clinical importance (see ref. 72 for review). As discussed above, the copy number status of a gene can theoretically be established by dPCR-based approaches with great precision in high-ctDNA scenarios (62); however, again at the expense of increasing number of assessed targets and a potentially high false negative rate when ctDNA is low. Finally, for some cancers, the absence of high frequency driver mutations such as prostate cancer, where the *SPOP*, mutated in 13% of patients is the most frequently

mutated gene (73), makes the development of such targeted panels even less practical.

Analyzing cfDNA using Massively Parallel Sequencing

The inherent limitations of targeted methods described above for determining more comprehensive mutational landscape of tumors prompted the development on more generalizable techniques. The necessity for development of such methods is further emphasized by the existence of intrapatient tumor heterogeneity—a well-documented phenomenon with relevance to treatment resistance and relapse (e.g., ref. 74). Improvements in read-length, sequence quality, and throughput allowed NGS methods to become a viable alternative for quantifying ctDNA. Limitations that remain in using NGS are the efficiency by which regions of interest can be captured/enriched from cfDNA and the higher error rate of sequencing relative to the accuracy of dPCR. A variety of strategies to drastically reduce the error rate of NGS for accurate ctDNA assessment have already been developed as will be discussed below. In general, current methods for using NGS in ctDNA quantification can be broadly divided into two groups. The first group relies on the amplification of the target regions using region-specific primers (often highly multiplexed), while the second relies upon hybridization-based capture of target regions using complementary oligonucleotides with subsequent amplification of the captured DNA (library). Both strategies are followed by highly redundant ("deep") sequencing to allow the relative amount of mutant and wild-type DNA molecules at each locus to be accurately counted.

PCR-based methods for cfDNA sequencing

A simple approach to amplify cfDNA for sequence-based characterization involves a PCR using site-specific primers with universal tails that facilitate library construction using nested PCR. An early example of a PCR-based strategy for analysis of mutations found in individual patients involved the design of a set of tailed site-specific primers followed by multiplex PCR (for preamplification) and subsequent uniplex PCR using each individual primer pair. The second PCR, in which locus-specific primers are applied individually, is accomplished using a Fluidigm AccessArray system and the entire procedure was named TAm-Seq (Tagged Amplicon Sequencing; ref. 75). This strategy can facilitate sequencing a panel of commonly mutated exons or may be guided by mutations identified through other means (e.g., genome or exome sequencing). This approach afforded the opportunity to quantify mutant DNA at many loci in each patient but had a relatively low sensitivity for mutations below approximately 1%–2%. Still, this pilot study showed clear evidence of a temporal correspondence between ctDNA levels and tumor burden when compared within individual patients. The multiplex nature of the assay also allows monitoring the level of many individual mutations in a single sample (or series) thereby allowing profiling cfDNA at each locus individually. Such distinct locus-specific profiles might be expected in patients with spatial heterogeneity of tumors or with ongoing clonal evolution in response to therapy. Anecdotal examples of this were first shown in a study by Dawson and colleagues, in which the level of mutant DNA corresponding to *TP53* and *PIK3CA* showed strikingly different dynamics across series of plasma samples from patients with metastatic breast cancer (34).

Until recently PCR-based methods were preferred for cfDNA analysis because they allowed sequencing of much smaller input DNA amounts. For example, in our own study of mCRPC patients treated with abiraterone and enzalutamide (31), we chose to combine targeted sequencing with whole-genome copy number profiling using array comparative genomic hybridization. Whole-genome copy number profiling allowed us to determine that pre-existing amplification of AR in this cohort was a marker of adverse outcome for patients switched onto enzalutamide. We successfully sequenced exon 8 that encodes part of the ligand-binding domain (LBD) of the AR gene from as little as 1 ng of input cfDNA. Out of six detected nonsynonymous LBD mutations, three were not previously observed in prostate cancer. Importantly, we also identified cases where a patient had multiple (up to five) mutations in the AR, while no DNA read, spanning the sequenced region, had more than two mutations. The most parsimonious explanation for that phenomenon is the existence of multiple tumor subclones, each with unique version of AR protein.

We have also observed changes in patients' AR LBD mutation landscape during the course of treatment. To understand the functional significance of these changes and to enable rational design of novel antiandrogens, we have established a resource for the functional characterization of all identified AR mutants. Therefore we characterized the effects of various steroids (DHT, estradiol, progesterone, and hydrocortisone) and different antiandrogens including enzalutamide and a novel agent developed at the Vancouver Prostate Centre (VPC) on transcriptional activity of the receptor. We established, *in vitro*, that all mutations detected in the plasma samples of mCRPC patients were resistant to at least one specific antiandrogen treatment and allowed us to explain some of the observed treatment-induced AR mutation landscape shifts. We also demonstrated that a novel AR inhibitor VPC-13566 under development at the VPC was able to efficiently target all tested AR mutants (76). To summarize, we have prototyped an analytic pipeline for evidence-based selection of optimal treatment strategies for mCRPC patients that may eventually enable rational and rapid selection of specific AR inhibitors to combat resistance.

In a broader application of this type of approach, Carreira and colleagues reported on the temporal sequential analysis of approximately 38-kb region using a custom Ampliseq panel (Thermo Fisher Scientific) from as little as 6 ng of cfDNA in a cohort of 16 *TMPRSS2-ERG*-positive prostate cancer patients (77). This work demonstrates the advantages of targeted sequencing approaches as a single assay enabled the detection of both CNAs and point mutations. Importantly, the authors also developed an approach for assessment of the abundance of ctDNA in total cfDNA, based either on the analysis of the allelic frequencies in monoallelic deletion regions, or in absence of those, on the comparison of read depths of the autosomal and nonautosomal regions in tumor and matched normal samples. This work also illustrated the main disadvantage of an amplicon-based targeted sequencing—varying PCR efficiency of the panel primer pairs. For example, for the copy number estimates the authors chose to retain only amplicons with read coverage falling in the range of mean \pm SD for more, than 10 samples. This resulted in discarding 35% (120/337) of autosomal amplicons (ref. 77; Supplementary Data). It also should be noted, that 6 ng of input DNA is arguably the lower practical limit of cfDNA input for sequencing as it corresponds to approximately 1,000 diploid genome equivalents. Assuming 1% ctDNA content, this amount would contain just 10

copies of tumor genomes—a level, likely to result in high sampling variance. Another key consideration when designing PCR primers for such experiments is that the distance between primers should correspond to the size of DNA fragments expected in ctDNA. In our experience, we have observed a bias towards PCR under-representing the level of ctDNA proportionally relating to the size of amplicons in accordance with observations made in the Diehl study (33). While keeping amplicon size to a minimum must be a priority (e.g., 60–80 bp) it should be also noted that this can be challenging for assays relying on hydrolysis probes, as little space is available in such a small region for two primers and a probe. Even in spite of such accommodations, any PCR-based method is expected to under-sample ctDNA owing to fragments in which both priming sites are not represented and this may be exacerbated in samples with contaminating DNA that can be less fragmented.

Ligation and hybridization-capture methods

A recently developed method for performing targeted sequencing is based on oligonucleotide DNA capture and has given rise to affordable approaches to sequence gene panels and even the entire human exome (78). This approach is based upon sequencing library construction followed by hybridization of the library to a pool of DNA or RNA oligonucleotides complementary to regions of interest. The hybrid molecules are then isolated (typically via immobilization on streptavidin beads) and amplified using universal primer pairs complementary to the library adaptors. Importantly, a convenient feature of such strategies that owes to the natural size distribution of ctDNA is that libraries can be generated using ligation-based chemistry directly without the need for shearing or transposon-based library construction. It is notable that in absence of shearing step much of the contaminating DNA from nonapoptotic processes is expected to be naturally excluded from libraries thereby naturally biasing the library contents in favor of true ctDNA fragments. Assuming sufficiently high ligation efficiency, this would result in a higher fraction of mutant ctDNA molecules making it through sequencing pipeline than PCR-based applications. This assumption has been experimentally validated in libraries prepared from cfDNA of hepatocellular carcinoma patients (79). The authors observed a shift towards the characteristic cfDNA fragment length in read pairs mapping in regions associated with copy number gains, where we would expect greater proportion of ctDNA-derived fragments. Despite this potential benefit, this does not preclude the need for standardization of blood collection and handling methods. Until recently, the major disadvantages of this type of approach to cfDNA analysis were relatively high requirements for the quality and quantity of input DNA. However, development of the solution-based hybridization workflows (80) and refinements in sequencing library construction protocols such as improvements of ligation efficiency allowed drastic reduction of the quality and quantity of input DNA requirements, resulting in the development of the capability to analyze DNA samples as scarce and highly fragmented as cfDNA.

One of the best examples of successful application of this strategy to development of a clinically relevant cfDNA-based analysis is the work of Newman and colleagues (81). In this seminal study, the authors first analyzed available sequence data to determine a set of genomic regions (a "selector") comprising a set of mutations present in majority of patients with stage II–IV

non-small cell lung cancer (NSCLC). Custom oligonucleotides covering these loci were purchased to allow hybridization capture of all DNA from these regions. They then combined a modified library construction strategy with sophisticated bioinformatics methods to sequence these loci in patient constitutional DNA, tumor, and cfDNA samples. Importantly, mutations identified using this method may include indels, single base substitutions, and breakpoints that underlie structural alterations such as those affecting *ALK* or large deletions. The latter types afford virtually perfect specificity for tumor DNA. Using the level of mutant DNA detected across the mutations found in the tumor, they demonstrated that cfDNA-based analysis allowed for earlier assessment of response to treatment than standard-of-care radiographic approaches and could distinguish between residual disease and treatment-related imaging changes, such as postradiotherapy inflammation. This so-called "CAPP-seq" method allowed analysis of as little as 7 ng of input cfDNA (~1,100 genome equivalents), essentially at the level of the best existing PCR-based target sequencing methods. This was a significant result, because it demonstrates the potential for hybridization-based workflows to compete with PCR-based strategies while allowing for a much broader and more uniform representation of the analyzed DNA than PCR-based techniques. We note that very inexpensive options now exist for obtaining individual capture oligonucleotides or pools that target the exons of a small number of genes such as biotinylated DNA LockDown oligonucleotides offered by Integrated DNA Technologies. This approach allows for flexibility in designing and modifying such selectors for individual patients or cohorts. For some tumor types, a smaller selector focusing on a small panel of exons may be suitable, whereas other may be better covered using a larger selector that includes genomic regions commonly affected by structural alterations. An important consideration when designing a selector is that sequencing cost is proportional to the size of the region being sequenced. It should be noted, that targeting very small regions is associated with its own hurdles because the enrichment efficiency of a single capture are typically on the order of 10^4 -fold, so for the targets smaller, than approximately 100 kb additional steps, such as two rounds of capture may be necessary to ensure sufficiently high on-target mapping rate (82). For patient-specific applications or cancers with a high mutation rate (or recurrence in a small number of genes), this may allow deep sequencing to be achieved at a low cost using "bench-top" NGS devices.

A logical extension of this approach that requires no prior knowledge of the tumor but assumes the presence of somatic point mutations affecting exons is the use of whole-exome sequencing. Early exploration of this strategy was demonstrated in six patients with a mixture of advanced breast, ovarian, and lung cancers by Murtaza and colleagues (83). Overall, a strong correspondence between the mutations was detected between the plasma and the matched tumor and the variant allelic frequency (VAF) was largely reflective of the level of ctDNA in each plasma sample. In almost all patients, at least one mutation was identified that showed an increase in VAF over the time of the study, and these included mutations in genes thought to be associated with treatment resistance in their respective disease such as *PIK3CA* in a breast cancer patient treated with paclitaxel. In another example, Butler and colleagues (84) compared tumor and plasma exomes from two patients with metastatic sarcoma and metastatic breast tumor. For sarcoma patients, 47 of 48 mutations identified in the tumor sample were also found by exome sequencing of the

cfDNA. However, for the patient with metastatic breast tumor the authors observed discordance for the H1047R *PIK3CA* mutation status. This mutation was detected in primary tumor but not in matched metastatic and cfDNA samples. *ESR1* mutation (D538G), on the contrary, was observed in both metastatic and cfDNA samples but not in tumor, and could possibly explain patient's resistance to estrogen deprivation therapy. The potential for observing discordance between tumor tissue and liquid biopsies via exome sequencing offers many avenues of research for studying the clonal complexity and patterns of evolution in cancer not previously readily accessible due to the invasive nature of tissue biopsies.

Importantly, despite some potential to observe tumor evolution and identify variants outside of gene panels or selectors in more targeted capture-based assays, exome sequencing remains a niche application. Large target size makes achieving the same level of coverage as for targeted gene sets impractical. Currently, that and the higher input DNA amount requirements (~ 100 ng for Butler and colleagues study; ref. 84), limits exome sequencing to the analysis of samples with relatively high ctDNA levels that would allow robust detection of mutations at the relatively shallow coverage of individual loci. In such scenarios, it is advisable to assess ctDNA first using a targeted approach to identify suitable samples such as those with VAFs of at least 5% at known mutant sites and high amounts of cfDNA in blood.

Error suppression methods

As we have demonstrated earlier, the mutant VAF in patients' cfDNA can go as low as 0.01%. Clearly, any strategy to suppress sources of errors thereby increasing accuracy in detecting mutant DNA molecules is important for cfDNA analysis. This problem is equivalent to an extreme case of identifying low-abundance mutations in tissue samples, which has also proven difficult for NGS methods. Guntry and Vijg noted that "the single most important limitation of current MPS approaches from mutation analysis is the inability to address low-abundance mutations that turn somatic tissues into mosaics of cells" (85). This argument was based on the comparison of the natural mutation rates in mammalian cells (e.g., 0.05×10^{-9} per base per cell division for human cells) versus well-established error rates of existing sequencing platforms, which was estimated in 2012 to be approximately 0.05%–1% and has not significantly changed since.

Besides simple strategies such as using proofreading-enabled polymerase and a low number of PCR cycles, the most promising strategy for error suppression is based on the fact that because DNA is naturally double-stranded, true mutations should be present in identical positions on both DNA strands from a single duplex. However, because all widely employed sequencing strategies now include a PCR amplification step, the real problem becomes distinguishing reads that originate from the same original duplex fragment (and thus should be identical) from reads that originate from multiple DNA fragments covering the same locus (that were produced from the other allele(s) or other cells in the analyzed mixture). One of the first solutions to this problem was proposed in 2011 by Kinde and colleagues. Their "Safe-Seqs" approach involves usage of pools of primers containing degenerate molecular tags that would uniquely barcode all DNA fragments resulting from the initial PCR cycle. The procedure is followed by a nested PCR to amplify each "family" of fragments that are later collapsed into consensus sequences. Interestingly, the authors provide hard data to assess the efficiency of their

approach. In one of the experiments, the authors used DNA from 1,750 individual cells to assess mutation frequency in one gene. Using endogenous UID approach, 1,057 molecules were assessed or approximately 30% of the total available amount (86). Comparable strategies for ligation-based library preparation with modified adaptors allow individual ligation events to be distinguished and even facilitate recognition of the two strands of the original duplex. Conventional methods to recognize reads deriving from the same template DNA molecule (i.e., duplicate pairs) are not suitable for ctDNA because the distribution of fragment ends is non-random and is likely dictated, in part, by nucleosome occupancy (87). As the read lengths and the robustness of paired-end sequencing of the massively parallel sequencing platforms improved, it became feasible to sequence approximately 200-bp fragments from both ends. Coupled with the molecular tagging, this opened the way to drastically decrease the sequencing pipeline error rate. For example, the authors of one of such approach, Schmitt and colleagues estimated the error rate of 3.8×10^{-10} in a model experiment (88). The same group demonstrated the robustness of their approach by detecting a single *ABL1* imatinib conferring mutation in a sample from the chronic myeloid leukemia patient (82). The E279K mutation was unambiguously detected at 1% rate, whereas the error rate of raw sequencing data would have either completely or partially obscured this mutation. In our own data, (Assouline and colleagues, in review) we have directly inferred mutations from ctDNA libraries using a similar error suppression strategy at levels as low as 1% across a broad gene panel. An interesting combination approach termed "integrated digital error suppression" (iDES) was described in 2016 by Newman and colleagues (89). The authors redesigned their previously published Capp-Seq panel (81) to integrate molecular barcoding into their workflow. However, analysis of the cfDNA sequencing results revealed the presence of particular sequencing artifacts in their cfDNA samples that were not suppressed by bar coding. This prompted the authors to rigorously examine the patterns of sequencing artifacts and note that G→T changes were much more prevalent than C→A changes. Moreover, this imbalance increased proportionately to an increase of the hybridization time. This allowed the authors to develop an *in silico* filter to remove this bias. The combination of *in silico* filtering and barcoding approaches allowed development of the workflow that had 15 times lower error rate than original CAPP-Seq approach.

An interesting alternative to barcoding techniques capitalized on the increased read length possible with newer Illumina chemistry and suggested the use of rolling circle amplification to ensure redundant sequencing of individual DNA fragments (90). Briefly, DNA is fragmented to approximately 130 bp, denatured, circularized, and amplified using Phi29 polymerase. Strand displacement activity of this polymerase ensures rolling circle amplification of individual DNA fragments. These can be sequenced on Illumina MiSeq machines using 500-cycle chemistry. As a result, each individual fragment of the original DNA mixture will be read on average three times. Interestingly, the authors demonstrated that redundant sequencing alone results only in approximately 2-fold reduction of error rate. More detailed analysis of the sequencing data revealed that most of the noise resulted from deaminated nucleotides (C→T and G→A transitions due to deamination of cytosine into uracil and of guanine into xanthine, respectively). Treating the circularized DNA fragments with uracil-DNA glycosylase and (UDG) and formamidopyrimidine-DNA-glycosylase (Fpg) resulted in excision of deaminated bases, and,

thus, in linearization of the fragments with such bases. Clearly, linearized fragments are excluded from subsequent rolling circle amplification and sequencing. This allowed the authors to achieve approximately 100-fold reduction in sequencing noise thereby improving specificity, but presumably at a cost to overall sensitivity due to the loss of some molecules prior to sequencing (90).

To summarize, all of the aforementioned approaches rely on redundant sequencing of individual DNA fragments to achieve drastic noise reduction and thus come at a higher cost than standard applications of DNA sequencing. Circle sequencing stands apart from other approaches due to lowest sequencing redundancy, but requires UDG/Fpg enzyme treatment step to ensure significant noise reduction. Another common problem for the barcoding approaches is their sensitivity to efficiency of library construction protocol, starting at ligation of barcoded adaptors and ending with final PCR amplification of selected DNA fragments. This number can be measured by calculating the ratio of the detected individual molecules after sequencing to the total approximate number of DNA molecules that enter the assay. Over last five years, this metric improved from around 30% in 2011 (86) to more than 60% in 2016 (89).

Commercialization efforts

The practical advantages of tumor monitoring from blood have spurred a number of commercialization efforts. To date, one of the most successful enterprises in this space is Guardant Health Inc. Its proprietary approach appears to be a variant of the barcoding method termed "digital sequencing" (91) based on the "nonunique" heptamer barcodes that tag both 5' and 3' ends of cfDNA fragments. The company claims that this process is 5–10 times more efficient than other existing workflows and that minor alleles at approximately 0.1% prevalence can be detected with extremely high specificity. The analysis workflow also allows for identification of the copy number gains, as long as the CN gain exceeds 2.2-fold in the cfDNA. Guardant Health offers a 54-gene panel for cfDNA-based screening of melanoma, lung, and breast cancer patients that allows both SNV detection and gene copy number evaluation. Other important players in this field including Personal Genome Diagnostics Inc. led by John Hopkins team that includes Drs. L. Diaz and V. Velculescu and Cambridge, MA based Foundation Medicine. The latter recently announced its own version of a barcoding assay that is also aimed at detecting low-level contamination in cfDNA samples.

One potential application of cfDNA that has been long envisioned is its use in identifying cancers prior to any symptoms, but owing to the difficulty and likely cost of tackling this problem, there are no studies yet demonstrating the feasibility of finding a solution. In reference to this "holy grail" application, in January 2016, Illumina Inc announced launch of GRAIL, a new company dedicated to developing and implementing cfDNA-based assay for the early detection of cancer in asymptomatic individuals. No details on the underlying technology have been released, beyond the statement that ultra-deep sequencing (20,000× or more) will be one of the cornerstones of the approach. A recent presentation for investors released by Illumina outlines two scenarios: one "best case" which assumes that GRAIL will develop a test suitable for high-risk individuals in whom cancers at higher stages (stage II and above) would be sought. Ultimately, they also plan to target patients in the general population using an approach suitable for early-stage cancer. In both cases, the analysis cost is expected to fall

in \$500–1,000 range. While the investor's presentation mentions "error-corrected reads", no details on the GRAIL's approach to error correction are available yet. To summarize, the liquid biopsy clearly represents an appealing area for commercialization. Thus far, the commercial success appears to be favoring companies that employ some form of error correction in their technologies, a trend we expect to continue.

Conclusion

The concept of precision oncology has as its foundation the ability to detect clinically relevant and actionable tumor-specific changes in a timely fashion. This may be achieved using temporal cfDNA assays to monitor adaptation to therapy and identify actionable mutations. cfDNA-based profiling of cancer patients offers a number of critical advantages for essentially real-time monitoring of a tumor response to therapy in cancer patients. These include integral representations of tumor heterogeneity, ease of sampling, minimal invasiveness and morbidity, and low cost. However, tumor-derived DNA usually constitutes only a small percentage of total cfDNA so the ability to detect rare genome aberrations is an essential requirement for cfDNA analysis pipelines. Another important parameter is the spectrum of genomic changes the technology is capable of detecting. While targeted assays can be fruitful in the clinical setting, sequence-based approaches offer clear advantages in terms of flexibility of coverage and the ability to detect a wide range of aberrations in tumor genomes. This flexibility will be especially important for managing metastasis and resistance to therapy; widely recognized to be among the most important problems in cancer management. Resistance to therapy can be driven by a wide range of genomic aberrations such as point mutations and copy number aberrations. Moreover, resistant subclones can constitute a very small proportion of the tumors total clonal population until the selective pressure of therapy leads to their rapid expansion. Clearly, the early detection of resistant clones requires sensitivity to detect such events. However, this requires minimizing noise in the cfDNA analyses and pushing the sensitivity of detection to the theoretical limits imposed by the plasma levels of cfDNA. Recent evidence suggests that the most promising technology for this is based on molecular tagging-based workflows that suppress errors introduced by PCR and sequencing. This approach is limited mainly by the cfDNA sampling efficiency and is straightforward to scale and thus offer enormous potential for monitoring cfDNA in cancer patients and possibly for screening healthy asymptomatic individuals.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Grant Support

R.D. Morin is funded by a New Investigator Award from the Canadian Institutes of Health Research (CIHR) and by the Terry Fox Research Institute (grant #1043) and a Terry Fox New Frontiers Program Project grant (#1021). C.C. Collins is supported by a Terry Fox New Frontiers Program Project grant #TFF116129, Prostate Cancer Canada – Movember Foundation Team grants D2013-4, D2015-06 the Terry Fox New Frontiers Program on Prostate Cancer Progression T2013-01, CCSRI Innovation grants 701115 and 701753, and CCSRI Impact grant 701585.

Received February 9, 2016; revised June 27, 2016; accepted June 30, 2016; published OnlineFirst July 15, 2016.

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**IN THE UNITED STATES DISTRICT COURT
FOR THE MIDDLE DISTRICT OF NORTH CAROLINA**

NATERA, INC.,

Plaintiff,

v.

C.A. No. 1:23-cv-629

NEOGENOMICS LABORATORIES,
INC.,

Defendant.

**PLAINTIFF NATERA’S REPLY IN SUPPORT OF ITS MOTION FOR A
PRELIMINARY INJUNCTION PURSUANT TO FEDERAL RULE OF CIVIL
PROCEDURE 65(A) AND 35 U.S.C. § 283**

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I. NATERA IS LIKELY TO PREVAIL ON THE MERITS

A. '454 Patent Infringement

NeoGenomics raises two non-infringement arguments: (1) RaDaR purportedly does not “sequenc[e] the amplicons” (Opp. 3-6); and (2) [REDACTED]

[REDACTED]

[REDACTED] (Opp. 6). Each fails.

1. “Sequencing the Amplicons”

NeoGenomics argues that “sequencing the amplicons” excludes additional amplification steps. But claims, like Claim 1, with “comprising” language can include unrecited steps precisely to avoid such “gotcha” theories of noninfringement. *Cias, Inc. v. All. Gaming Corp.*, 504 F.3d 1356, 1360 (Fed.Cir.2007). All Claim 1 requires is “sequencing the amplicons.” RaDaR *does* obtain sequence reads of the amplicons generated via targeted multiplex amplification, as required by this “sequencing” step. Metzker.Decl., § IX.A, ¶270.

NeoGenomics’ contrary argument (a disguised claim construction) is that “amplicons”¹ from targeted amplification must be “directly” sequenced without a modifying intervening step. Van-Ness.Decl², ¶78; Van-Ness.Tr.³, 236:21-22. But

¹ NeoGenomics never proposed—therefore waived—a construction for this term. *Adv. Comm’n Design, Inc. v. Premier Retail Networks*, 46 F.App’x 964, 980 (Fed.Cir.2002).

² Dkt. 116.

³ See Metzker.Decl., Ex. 1.

accepting this requires improperly reading the term “direct” into Claim 1, and disregarding controlling precedent, established claim construction canons, and the ’454 Patent’s teachings. Metzker.Decl., § IX.A, ¶¶271-272.

For example, asserted Claim 11, which depends from Claim 1, introduces an additional step of “barcoding PCR prior to the sequencing.” Metzker.Decl., ¶¶273-274. Under the doctrine of claim differentiation, Claim 1’s scope is broader than Claim 11—thus additional PCR steps are permitted, though not required. *AK Steel Corp. v. Sollac*, 344 F.3d 1234, 1242 (Fed.Cir.2003). NeoGenomics’ Opposition fails to address Claim 11.

The ’454 specification teaches additional amplifications following targeted amplification, (*e.g.*, Example 7), and sequencing using Illumina’s system, which involves adding universal sequencing adapters by PCR, (Metzker.Decl., ¶274). NeoGenomics’ interpretation excludes these necessary steps and reads out preferred embodiments which is “rarely, if ever, correct.” *Vitronics Corp. v. Conceptronic, Inc.*, 90 F.3d 1576, 1583 (Fed. Cir. 1996).

NeoGenomics’ reading also contradicts the meaning of “amplicons.” PCR-related terms such as “amplicons” and “primers” refer to functional units of DNA involved in the PCR process—molecules containing the same characteristic sequence, which can be modified as needed. Contrary to NeoGenomics’ characterization⁴, when a similar argument arose in *ArcherDx*, the Court—and jury—rejected requiring identity between “a universal

⁴ Dkt. 107 at 3.

primer” and “the universal primer” in a claim. That the same *sequence* was present in the two was sufficient for infringement.

NeoGenomics cites supposed “admissions” made during prosecution of a different patent, U.S. 11,486,008. Opp. 4-5. But absent allegation that a patentee acted as his own lexicographer (here there is none), claim scope can only be surrendered where there is a disavowal in the specification or during prosecution, and NeoGenomics does not argue one occurred. *AstraZeneca LP v. Breath Ltd.*, 542 F. App’x 971, 975-76 (Fed.Cir.2013) (disclaimer must be “clear and unmistakable”); Metzker.Decl., §IX.A.iii, ¶292. Moreover, because the ’454 and ’008 claims have materially different language⁵ and the PTO found them patentable based on different arguments, statements in the ’008 prosecution do not limit the ’454 claims. *Invitrogen v. Clontech Labs.*, 429 F.3d 1052, 1078 (Fed.Cir.2005); *ResQNet.com v. Lansa*, 346 F.3d 1374, 1383 (Fed.Cir.2003).⁶

Even if NeoGenomics’ construction were adopted, RaDaR would still infringe under doctrine of equivalents. *Spectrum Pharm. v. Sandoz*, 802 F.3d 1326, 1337

⁵ The ’008 claims as issued recite sequencing “the amplicons obtained in the multiplex targeted amplification reaction,” language which does not appear in any ’454 claim. Metzker.Decl., ¶.

⁶ NeoGenomics’ cited cases are inapposite. (Opp. 5) *Elkay Mfg. Co. v. Ebco Mfg. Co.*, involved two sets of claims the applicant “affirmatively linked” during prosecution, and the issued claim language was identical but for one term. 192 F.3d 973, 980 (Fed. Cir. 1999). *Juniper Networks, Inc. v. Palo Alto Networks, Inc.*, addressed doctrine of equivalents where (unlike here) the asserted patent was a direct continuation of the earlier patent, and the Examiner allowed the claims for “substantially similar reasons,” linking them. 15 F. Supp. 3d 499 (D. Del. 2014). The ’454 and ’008 Patent claims differ in materials ways (not by a mere word) and were never expressly linked.

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B. '035 Patent Infringement

Regarding Claim 1, NeoGenomics argues—incorrectly—that RaDaR does not perform targeted amplification of tagged products. Opp. 6-7. RaDaR allegedly does not infringe because, while it uses targeted PCR to generate tagged products, it does not perform a subsequent targeted PCR to separately amplify those tagged products. Opp. 7. Claim 1, however, does not require two separate targeted PCRs for “tagging” and “amplifying.” Metzker.Decl., §IX.B., ¶297.

PCR involves of a series of amplification “cycles,” each involving a separate and new amplification of what was produced in prior cycles. Van-Ness.Tr. 220:1-16; Metzker.Decl., §V.B.ii-iii. RaDaR’s targeted PCR involves 15 cycles. Metzker.Decl., ¶295; Van-Ness Tr. 228:17-19. The first cycle(s) of RaDaR’s targeted PCR introduces universal tags, one on each strand of the resulting product (“tagging” step). Metzker.Decl., ¶296. NeoGenomics argues that the initial tagging occurs over two cycles, (Opp. 7), but even if true, that would be irrelevant. All agree at least one tag is added at the first cycle, the dual-tagged products are formed by at least the second cycle’s end, and tagged products

are thereafter amplified by targeted amplification numerous times in the remaining cycles of PCR (“amplifying” step). Metzker.Decl., §IX.B; Van-Ness.Tr. 228:20-232:25.

C. NeoGenomics Has Not Raised A Substantial Question Of Invalidity

1. Obviousness

NeoGenomics does not argue the Natera claims lack novelty. NeoGenomics argues they are obvious. Opp. 8-11. Obviousness, however, requires (1) motivation to combine and (2) reasonable expectation of success. *Regents Univ. Cal. v. Broad Inst.*, 903 F.3d 1286, 1291 (Fed.Cir.2018). NeoGenomics makes neither showing.

NeoGenomics and its expert identify no evidence of motivation to combine. *See generally* Van-Ness.Decl., §X; Metzker.Decl., ¶75. That alone defeats NeoGenomics’ arguments. *In re Rouffet*, 149 F.3d 1350, 1358 (Fed.Cir.1998).

NeoGenomics also fails to grapple with unpredictability of the art, particularly well-known obstacles to the use of cell-free DNA (“cfDNA”), which is challenging to manipulate even with known methods. Metzker.Decl., §II.A; Van-Ness.Tr., 109:25-110:7, 116:19-117:21. In combining disparate teachings, Dr. Van Ness fails to address—even acknowledge—difficulties in identifying genetic variants in low-abundance, unpredictable cancer-associated cfDNA (“ctDNA”). As skilled artisan would not be motivated to modify the prior art to achieve the claimed methods or reasonably expect success doing so. NeoGenomics’ obviousness combinations all fail. 903 F.3d at 1291.

NeoGenomics’ technical arguments also lack merit. **First**, the ’454 claims are not obvious over Forsheo (Opp. 10), as the Examiner recognized during prosecution.

Metzker.Decl., §VI.A.i.a.C. Sequencing each SNV locus 50,000 times or more—which Forshew does not teach, *id.*— would not reasonably be expected to identify rare variants in low-abundance ctDNA above the background “noise” from sequencing-related error rates. *Id.* §VI.A.i.a.B.

Second, it would not have been obvious to modify Bashashati⁸ to amplify multiple cfDNA loci in one reaction volume. (Opp. 10). Doing so could generate unwanted byproducts and hamper the method’s success. Metzker.Decl., §VI.A.ii.a; Van-Ness.Tr. 132:13-133:3.

Third, the ’035 Patent is not obvious over Kaper’s Access Array system (Opp. 10). Metzker.Decl., §VI.B.i. The ’035 Patent *teaches away* from using Access Array, disparaging it as “problematic for samples with a limited amount of DNA”—*e.g.*, ctDNA. *Id.* §VI.B.i.a.

Finally, NeoGenomics’ reliance on ARM-PCR fails to teach cfDNA or targeted amplification of SNP loci in one reaction volume. *Id.* §§VI.B.ii.a-b.

2. Written Description

Contrary to NeoGenomics’ argument (Opp. 11-12), the ’454 specification describes whole genome sequencing (“WGS”) explicitly and in terminology a skilled artisan would recognize—*e.g.*, “long read sequencing” and “phasing.” Metzker.Decl., §VIII.A; *Ariad*

⁸ Dkt. 118-14 (’454 Patent, p.8).

Pharms., Inc. v. Eli Lilly & Co., 598 F.3d 1336, 1352 (Fed.Cir.2010) (written description “does not demand any particular form of disclosure”).

NeoGenomics’ other conclusory arguments (Opp. 12) lack merit because the patents show possession of the inventions and provide examples. Metzker.Decl., §VIII.B. The patents teach the claimed methods could be performed without selecting primers to avoid “primer dimer” formation. *Id.* §VIII.B.i.

3. Inventorship

NeoGenomics’ inventorship argument (opp. at 11-14) is a red herring. NeoGenomics cannot overcome the presumption that inventorship is accurate. *Hess v. Adv. Cardiovascular Sys.*, 106 F.3d 976, 980 (Fed.Cir.1997). There is no evidence any allegedly omitted inventor contributed to **conception** of the ’454 claims. *Ethicon, Inc. v. U.S. Surgical Corp.*, 135 F.3d 1456, 1460-61 (Fed.Cir.1998). At most, NeoGenomics suggests Dr. Jamal-Hanjani articulated the state of the prior art regarding “whole exome sequencing” (WES) to Natera, but that is not enough to be an inventor. *See id.*, *Eli Lilly and Co. v. Aradign Corp.*, 376 F.3d 1352, 1359 (Fed. Cir. 2004); Van-Ness.Tr 22:17-24:1.

[REDACTED]

[REDACTED] And Natera already conceived of using WES in the context of the ’454 claims by June 2014, months before meeting Dr. Jamal-Hanjani. Ex. 1 (NAT-NEO-00884450); Ex. 2 (NAT-NEO-00574120-22); Ex. 3 (NAT-NEO-00774814, -21, -25); Ex. 4 (Zimmermann.Tr) 109:24-113:19; 100:24-104:8, 105:16-109:13.

Improper inventorship is rarely grounds for denying a preliminary injunction; it can be corrected any time—including during litigation and without explanation—without affecting validity. Stoll.Decl., §VI.D; *Egenera v. Cisco Sys.*, 972 F.3d 1367, 1376 (Fed.Cir.2020); *Canon Computer Sys. v. Nu-Kote Int'l*, 134 F.3d 1085, 1088 (Fed.Cir.1998). Natera did so during prosecution of the '035 Patent,⁹ and during litigation asserting its ancestor '220 Patent, which was upheld in *Natera v. ArcherDx* despite the same inventorship challenge. Dkt. 109-1 at 10-11.

4. Section 101

Natera's response to NeoGenomics' motion to dismiss (Dkt. 131), incorporated by reference, explains why the Natera Patent claims are patent-eligible. *See also* Metzker.Decl., §VII.

II. NATERA WILL SUFFER IRREPARABLE HARM

A. Natera Did Not Unreasonably Delay

NeoGenomics' "delay" argument (Opp. 15) fails. Delay does not bar an injunction where, as here, there is good cause. *Hybritech v. Abbott Labs.*, 849 F.2d 1446, 1457 (Fed.Cir.1988). Natera has been litigating to enforce its patents since 2020, and these asserted patents did not issue until December 2022. *See* D.Del. case nos. 20-cv-38, 20-cv-125, 21-cv-56. A patentee facing multiple infringers will often have "good cause for seeking relief against [a different infringer] first." *Hybritech*, 849 F.2d at 1457; *Polymer Techs. v. Bridwell*, 103 F.3d 970, 975-76 (Fed.Cir.1996).

⁹ Stoll Decl. ¶¶54-56.

Further, before July 2023, RaDaR lacked Medicare approval. NeoGenomics contends (Opp. 16) that Medicare approval does not drive sales, citing a survey. Dkt. 110-1. That survey found, however, that “reimbursement status” was an important factor influencing MRD-test-vendor choice, ranking higher than “cost,” “report clarity/comprehensiveness,” and “quality of customer support.” *Id.*, NEOGEN4241; *see* Ex. 5., Malackowski.Tr., 71 [REDACTED] NeoGenomics itself has made a point to publicly announce RaDaR’s Medicare approval for RaDaR.¹⁰

B. Natera Will Lose Market Share, First-Mover Advantage, Clinical Opportunities, and Biopharmaceutical Partnerships

NeoGenomics’ argument (Opp., 17) that there is no irreparable harm because of Natera’s large market share is misplaced. A patentee’s large market share weighs *in favor* of an injunction—not against—because the patentee has more to lose from infringement than the infringer has from injunction. *Metalcraft of Mayville v. Toro*, 2016 WL 4076894, *4 (E.D.Wis.2016) (granting PI to patentee with “about 100% of the market”); *Arlington Indus. v. Bridgeport Fittings*, 2011 WL 2927817, *10 (M.D.Pa. July 18, 2011) (similar). Even the report NeoGenomics cites predicts *Natera will lose* and *NeoGenomics will gain* market share. Dkt. 92-1, NEOGEN00004203. Further, NeoGenomics cites only data for

¹⁰ Dkt. 1-30 at 2.

the overall MRD-test market, not the tumor-informed market, [REDACTED]

[REDACTED].¹¹

NeoGenomics incorrectly contends that “Natera and NeoGenomics do not directly compete in the MRD market.” Opp. 18. [REDACTED]

[REDACTED]

[REDACTED]

Malackowski.Tr., 60-62, 71; Dkt. 12-23, 12. The implausibility of NeoGenomics’ position is illustrated by its contentions that: (1) Natera will not be harmed because Signatera has “numerous competitors” (Ex. 6 at 3), and (2) [REDACTED]

[REDACTED]. Malackowski Tr., 73-76.¹²

Natera’s CEO’s statements that Guardant’s *tumor-naïve* MRD test is Natera’s largest competitor and new competitors have “got a very long way to go” (Opp., 18-19) reflect only that Natera holds the largest share of the *overall* MRD-test market. They do not suggest RaDaR’s entrance into the tumor-*informed* market will not harm Natera.

¹¹ NeoGenomics’ contention (Opp., 24) that injunction “would change the status quo, not preserve it” because RaDaR will lose ill-gotten market share is contrary to law. *Atlas Powder v. Ireco Chems.*, 773 F.2d 1230, 1231 (Fed.Cir.1985).

¹² That the parties are the only two significant players in the tumor-informed market is alone sufficient for irreparable harm. *Douglas Dynamics v. Buyers Prods.*, 717 F.3d 1336, 1345 (Fed.Cir.2013); *Robert Bosch v. Pylon Mfg.*, 659 F.3d 1142, 1151 (Fed.Cir.2011).

Nor does the law require specific lost contracts.¹³ Notwithstanding, [REDACTED]

[REDACTED] (Sikri.Tr., 134) and Natera identified a lost contract with Moderna. Dkt. 6, 18. NeoGenomics ignores that: (1) Moderna partnered with NeoGenomics to use its tumor-informed MRD test, RaDaR; (2) [REDACTED] [REDACTED] (Malackowski.Tr., 60); and (3) because of RaDaR, Moderna no longer needed Natera.

Nor has Natera licensed the patents to Clariant. Opp. 21. That license expired in 2018, four years before the asserted patents issued. Even if that license included the patents, “the fact that a patentee has licensed others under its patents does not mean that unlicensed infringement must also be permitted while the patents are litigated.” *Abbott Labs. v. Sandoz*, 544 F.3d 1341, 1361-62, (Fed.Cir.2008).¹⁴

C. Natera Will Suffer Reputational Harm

Rather than dispute it is harming Natera’s reputation, NeoGenomics slings mud. Opp., 22. That third parties have injured Natera’s reputation does not preclude further injury by NeoGenomics. Nor has NeoGenomics shown these third-party statements are independent of its own conduct because many of them occurred *after* NeoGenomics began

¹³ See *Apple v. Samsung Elecs.*, 2012 WL 2576136, at *5 (N.D.Cal. July 3, 2012) (“the Federal Circuit has not required proof of specific lost customers”) (citing *i4i Ltd. P’ship v. Microsoft*, 598 F.3d 831, 862 (Fed. Cir. 2010)); *Canon v. Color Imaging*, 292 F.Supp.3d 1339, 1347 (N.D.Ga.2018).

¹⁴ NeoGenomics cites *ActiveVideo Networks v. Verizon Communications*, 694 F.3d 1312, 1338 (Fed.Cir.2012), but those parties did not compete and the patentee tried to license the patent throughout the industry, including to the defendant.

infringing and disparaging Signatera. Infringement is also depriving Natera of opportunities to *improve* its reputation because [REDACTED]

[REDACTED] Malackowski.Tr., 121.

D. There Is Causal Nexus

NeoGenomics contends RaDaR's sales are driven by sensitivity and advanced bioinformatics, not Natera's patents. Opp. 24 (citing Dkt.115 ¶44). But the cited declaration paragraph simply opines RaDaR is sensitive, not that sensitivity drives demand. RaDaR's sensitivity is irrelevant because the law requires "only "some connection between the patented feature and demand for" RaDaR, not that "a patented feature is the one and only reason for consumer demand." *Apple v. Samsung Elecs.*, 735 F.3d 1352, 1364 (Fed.Cir.2013).

III. BALANCE OF HARMS

Contrary to NeoGenomics' contention (Opp., 24), the fact that an injunction may harm its market share or customer relationships is irrelevant. *See Pfizer v. Teva Pharms., USA*, 429 F.3d 1364, 1382 (Fed.Cir.2005); *Celsis In Vitro v. CellzDirect*, 664 F.3d 922, 931 (Fed.Cir.2012). NeoGenomics' expert and 30(b)(6) deponent [REDACTED]

[REDACTED]. Malackowski.Tr., 121-22. Nor does NeoGenomics' investment in purchasing Inivata (RaDaR's developer), [REDACTED]

[REDACTED] work in its favor. *i4i*, 598 F.3d at 863 (rejecting defendant's reliance on development costs); Malackowski Tr., 127.

IV. PUBLIC INTEREST

NeoGenomics does not dispute that Natera has capacity to meet increased demand, weighing in favor of an injunction. *Boehringer Ingelheim Vetmedica v. Schering-Plough*, 106 F.Supp.2d 696, 707 (D.N.J.2000). NeoGenomics’ unproven claims that RaDaR has “high sensitivity” and is superior are undermined by its argument that RaDaR will not capture a large portion of the market. *Target Therapeutics v. SciMed Life Sys.*, 1996 WL 289034 (N.D.Cal.1996) (granting injunction despite argument that physicians preferred defendant’s catheters; finding patentee’s 85% market share undermined defendant’s claim to product superiority), *vacated on other grounds* 113 F.3d 1256 (Fed.Cir.1997). Nor would an injunction “interfer[e] with prospective trials” or “hurt on-going cancer research” because of the carve-out in Natera’s proposed injunction for existing patients. Dkt. 5-1 at 2.¹⁵ NeoGenomics’ declarant, Mr. Sikri, opines [REDACTED] [REDACTED] (Dkt. 115 at ¶¶15, 31-34, 39, 43, 47), but its expert and 30(b)(6) deponent [REDACTED] (Malackowski.Tr., 136).

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¹⁵ *Cordis Corp. v. Boston Scientific Corp.*, 99 F.App’x 928, 935 (Fed.Cir.2004), and *Hybritech*, 849 F.2d at 1458, are not contrary. *Hybritech* affirmed without explanation a record-specific finding. *Id.* In *Cordis*, the patentee could not satisfy demand and its product had “safety or efficacy concerns.” 99 F.App’x at 935.

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CERTIFICATE OF WORD COUNT

The undersigned counsel hereby certifies that this brief complies with the 3,125-word count limitation of Local Rule 7.3(d).

November 6, 2023

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CERTIFICATE OF SERVICE

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EXHIBIT 5

(Filed Under Seal)

IN THE UNITED STATES DISTRICT COURT
FOR THE MIDDLE DISTRICT OF NORTH CAROLINA

NATERA, INC.,)
)
Plaintiff,)
)
-vs-) C.A. No. 1:23-cv-629
)
NEOGENOMICS LABORATORIES, INC.,)
)
Defendant.)

** CONFIDENTIAL - OUTSIDE COUNSEL EYES ONLY **

Video-recorded Rule 30(b)(6) deposition of
NEOGENOMICS LABORATORIES, INC., by and through its
corporate representative JAMES E. MALACKOWSKI, and JAMES
E. MALACKOWSKI, in his personal capacity, taken before
TRACY L. BLASZAK, CSR, CRR, and Notary Public, pursuant
to the Federal Rules of Civil Procedure for the United
States District Courts pertaining to the taking of
depositions, at Suite 980, 190 North Wacker Drive, in
the City of Chicago, Cook County, Illinois at 12:00 p.m.
on the 24th day of October, A.D., 2023.

Page 1

Veritext Legal Solutions
Calendar-CA@veritext.com 866-299-5127

1 refer to tumor informed as a market, tumor naïve as a 13:25:42	1 the purpose of citing to this. 13:29:01
2 market, et cetera. 13:25:46	2 As I indicated within my report, I certainly 13:29:03
3 Q Products within a market by definition compete 13:25:47	3 make reference to when RaDaR was launched. And there is 13:29:05
4 with one another, right? 13:25:49	4 no need for me to replicate that here. It's not 13:29:11
5 A No, they do not. They are going after the same 13:25:51	5 something I'm trying to avoid in any way. It's not the 13:29:15
6 end customer, but that doesn't mean that they are 13:25:54	6 point of this paragraph which is "admitted lack of 13:29:19
7 competitive. 13:25:57	7 direct competition." 13:29:22
8 So, for example, a Ferrari and a Hyundai are in 13:25:59	8 Q This document is dated June 6th of 2023, right? 13:29:23
9 the same market of automobiles, but I would say they 13:26:03	9 A Yes. 13:29:29
10 very rarely, if ever, compete. 13:26:06	10 Q Between March of 2023 and June 6 is, what, less 13:29:33
11 Q You see also on page 6 this document states, 13:26:37	11 than three months, two or three months? 13:29:37
12 "Management says Natera has seen limited competition 13:26:40	12 A Yes. 13:29:40
13 from Inivata and NeoGenomics to date"? 13:26:44	13 Q How much competition would Natera be expected to 13:29:40
14 A I'm sorry, can you focus me on which paragraph? 13:26:49	14 see in two or three months after the launch of RaDaR? 13:29:43
15 Q Sure. We're on page 6, right? 13:26:52	15 MR. NOLAN: Objection. 13:29:47
16 A Yes. 13:26:54	16 THE WITNESS: I wouldn't expect Natera to see any 13:29:52
17 Q The first paragraph of the first section, not 13:26:54	17 competition because of their market dominance and the 13:29:54
18 the title, maybe line 6 down, first word, "Management." 13:26:57	18 product distinctions between the Natera product and the 13:30:00
19 A I see. 13:27:01	19 NEO product as described within my report. 13:30:04
20 Q You quote this in your report, don't you? 13:27:01	20 MR. SMITH: Q Assuming there are no -- were no 13:30:07
21 A I do. 13:27:06	21 product distinctions between a new tumor-informed MRD 13:30:10
22 Q And that's -- but in your report you do not 13:27:08	22 product and Signatera and that new product is launched 13:30:15
23 include the remainder of this sentence which states, 13:27:12	23 in March of 2023, how much competition would Signatera 13:30:19
24 "Though NEO's RaDaR was only launched in March 2023." 13:27:14	24 be expected to see by June 6th, 2023? 13:30:23
Page 54	
1 A Well, I make several references to this concept 13:27:21	1 A Well, that's a hypothetical that doesn't exist 13:30:27
2 starting with, I believe, Mr. Chapman's testimony or 13:27:25	2 because I think the evidence does suggest that there is 13:30:29
3 public statements. And then I affirm that the analyst 13:27:29	3 a difference. 13:30:32
4 conformed to that. I don't recall if I included that 13:27:33	4 But putting that aside an accepting your 13:30:33
5 last phrase, though I certainly don't dispute it. I 13:27:37	5 hypothetical assumption, I don't think the evidence of 13:30:34
6 describe within my declaration exactly when RaDaR was 13:27:43	6 this case would suggest that they would still see any 13:30:37
7 launched. 13:27:46	7 competition because of the size of the market and the 13:30:41
8 Q Well, just for the record, this is paragraph 56 13:27:46	8 relatively low penetration by Natera and everyone else 13:30:44
9 of your declaration where the quote appears. Why did 13:27:49	9 combined. 13:30:51
10 you not include the remainder of the quote where it 13:27:58	10 There is a lot of virgin territory for a new 13:30:52
11 states, "though NEO's RaDaR was only launched in March 13:28:00	11 competitor to address in a way that wouldn't affect 13:30:55
12 2023"? 13:28:04	12 Natera and, in fact, they would be economically incented 13:30:59
13 MR. NOLAN: I'm going to object to that question. 13:28:10	13 to do just that. Why go compete directly with someone 13:31:04
14 MR. SMITH: What's the basis for the objection? 13:28:13	14 when you can just go to unmet need where there is no 13:31:10
15 MR. NOLAN: There is a factual error in the 13:28:18	15 direct competition. 13:31:13
16 question. 13:28:21	16 Q What is that virgin territory you are referring 13:31:14
17 MR. SMITH: What's the factual error? 13:28:22	17 to? Who are these patients? 13:31:18
18 MR. NOLAN: That RaDaR was only launched in March of 13:28:24	18 A It's the representation of the \$20 billion MRD 13:31:20
19 2023 as is outlined in the declaration. 13:28:26	19 market. 13:31:23
20 MR. SMITH: Q Do you remember the question? 13:28:39	20 Q So is it your position that there is, these 13:31:23
21 A Yes. 13:28:43	21 patients are out there that are in need of an MRD test 13:31:28
22 My quote from Exhibit 4 is simply a short 13:28:51	22 that are not receiving any MRD test? 13:31:33
23 phrase noting that third party research reports confirm 13:28:53	23 A Well, I think that's probably factually true, 13:31:35
24 what Mr. Chapman has publicly admitted. And so that's 13:28:57	24 but the market is transitioning to service them. So 13:31:37
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<p>1 there is a conversion happening now that is causing all 13:31:42 2 of the participants to experience increased sales. 13:31:44 3 Q Is it your position that the patients that 13:31:50 4 receive a RaDaR MRD test if they were not to receive 13:31:52 5 that RaDaR MRD test would not receive any MRD test at 13:31:56 6 all? 13:32:00 7 A There is no evidence to suggest that their 13:32:01 8 physicians were considering another test as an 13:32:04 9 alternative. I've seen no evidence to suggest that 13:32:06 10 there was direct competition for a given account. And 13:32:10 11 so the evidence I've seen suggests that physicians are 13:32:13 12 adopting for the first time this technology, in part, 13:32:16 13 because of the unique characteristics of NEO. 13:32:20 14 Q When a doctor adopts MRD technology for the 13:32:24 15 first time, he has to choose an MRD vendor, correct? 13:32:27 16 A If that choice is available. 13:32:33 17 Within my report, for example, I talk about the 13:32:36 18 difference in the stage of medical attention and whether 13:32:38 19 or not it is the adjuvant stage or the surveillance 13:32:45 20 stage. So if the doctor is in the adjuvant stage, they 13:32:47 21 don't have NEO as a logical choice, so they basically 13:32:55 22 use Natera. 13:32:58 23 Q Have you ever heard a doctor say -- Let me 13:33:00 24 rephrase. 13:33:02</p> <p style="text-align: right;">Page 58</p>	<p>1 A I don't know. I would have to sit down and try 13:34:37 2 to make some calculations. 13:34:39 3 Q But you believe it's above 90 percent at least? 13:34:41 4 A For tumor informed? 13:34:44 5 Q Yes. 13:34:47 6 A Yes, currently. 13:34:47 7 Q Do you know what percentage of tumor-informed 13:34:48 8 MRD testing within the United States is currently 13:34:49 9 supplied by NeoGenomics? 13:34:52 10 A Well, my understanding is it would be 13:34:55 11 substantially the residual not supplied by Natera, so 13:34:57 12 something less than 10 percent. 13:35:02 13 Q When you say the residual, basically 100 percent 13:35:07 14 minus whatever Natera's percent is, is that what you're 13:35:11 15 saying? 13:35:15 16 A Yes. There may be some minor players who are 13:35:15 17 merging and have some initial penetration, but, 13:35:18 18 essentially, at this point it would be either Natera or 13:35:21 19 NEO for tumor informed. 13:35:26 20 Q Are you aware of any market share predictions 13:35:31 21 relating to a tumor-informed MRD market? Have you seen 13:35:33 22 any of those? 13:35:37 23 A Well, we reviewed certain market share 13:35:38 24 predictions earlier. 13:35:41</p> <p style="text-align: right;">Page 60</p>
<p>1 Are you aware of any doctor who has used a 13:33:03 2 RaDaR MRD test say had RaDaR not been available, I would 13:33:08 3 not have used any MRD test at all? 13:33:13 4 A I don't recall a doctor using those specific 13:33:15 5 words. [REDACTED] 6 [REDACTED] 7 [REDACTED] 8 [REDACTED] 9 Q Do you know what percentage of tumor-informed 13:33:48 10 MRD testing within the United States is performed by 13:33:50 11 Natera? 13:33:53 12 A The vast majority. I think it's like -- cited 13:34:00 13 within my report. But of the sales that are made, they 13:34:05 14 have approximately 70 percent or so of those sales. 13:34:07 15 Q That's a percentage for tumor informed or MRD 13:34:11 16 tests generally? 13:34:13 17 A MRD tests generally. It would be much higher 13:34:15 18 for tumor informed. 13:34:17 19 Q Do you have an estimate for tumor informed 13:34:20 20 specifically? 13:34:22 21 A No, I don't present such an estimate, no. 13:34:26 22 Q Do you think it's above 90 percent? 13:34:30 23 A I would expect it to be. 13:34:32 24 Q Above 95 percent? 13:34:34</p> <p style="text-align: right;">Page 59</p>	<p>1 And just to my last answer, we also do have to 13:35:42 2 take into account Invitae and to the extent that their 13:35:45 3 product is also within the same market space. 13:35:51 4 Q Well, let's go back, then. What percentage of 13:35:57 5 the tumor-informed MRD market does Invitae have? 13:36:01 6 A I don't recall. 13:36:06 7 Q Do you know how an oncologist goes about 13:36:10 8 deciding whether to order an MRD test? 13:36:12 9 A Are you asking me logistically or from a 13:36:16 10 decision standpoint? 13:36:19 11 Q From a decision standpoint. 13:36:21 12 A It's based upon their knowledge of what's 13:36:23 13 available in the market and their particular patient 13:36:25 14 condition and their judgment as to whether or not such a 13:36:27 15 test would be cost beneficial. 13:36:30 16 Q When oncologists consider whether it would be 13:36:35 17 cost beneficial, do they take into account whether there 13:36:39 18 is insurance coverage for the MRD test? 13:36:41 19 A As a sixth or seventh consideration in their 13:36:47 20 decision making, not as a primary consideration. 13:36:50 21 Q What factors influence an oncologist's decision 13:37:04 22 when choosing which MRD test to order? 13:37:10 23 A I think it's the same factors I noted before, 13:37:13 24 their knowledge of what's available, the patient's 13:37:15</p> <p style="text-align: right;">Page 61</p>

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1 condition. 13:37:18	1 THE WITNESS: I would think that would depend upon 13:39:51
2 When I talk about cost benefit, too, I wouldn't 13:37:18	2 the given oncologist and what research and education 13:39:52
3 limit that to dollar cost. There is certain other 13:37:20	3 they've been given. I could not speak to that 13:39:55
4 expenses to the patient in terms of lifestyle, having to 13:37:24	4 generally. 13:39:57
5 have additional blood work taken, the patient's interest 13:37:28	5 MR. SMITH: Q If an adequate tissue sample is not 13:39:57
6 in receiving interim results and knowing that. All of 13:37:33	6 available from the patient, is a tumor-informed MRD test 13:40:01
7 that goes into the doctor's decision. 13:37:37	7 an acceptable alternative to a tumor-naïve MRD test? 13:40:05
8 Q Does whether the MRD test is tumor informed or 13:37:41	8 MR. NOLAN: Objection. 13:40:11
9 tumor naïve affect -- influence an oncologist's decision 13:37:43	9 THE WITNESS: Could you repeat that question. I 13:40:12
10 about which test to purchase? 13:37:49	10 don't know if you misspoke or I just misheard it. 13:40:13
11 A Well, to the extent that a tumor-informed option 13:37:52	11 MR. SMITH: Q Let's imagine a hypothetical where 13:40:13
12 is no longer available because there is no tumor 13:37:55	12 the patient, there is no tissue sample available. You 13:40:15
13 samples, it would effectively be a consideration. 13:37:58	13 with me? 13:40:18
14 But the evidence I've seen in this case is that 13:38:01	14 A Okay. 13:40:20
15 doctors aren't making a decision primarily upon that 13:38:04	15 Q The doctor in that situation, could he order a 13:40:20
16 distinction. They're making it based upon sensitivity 13:38:08	16 tumor-informed MRD test? 13:40:23
17 and other aspects of the test. 13:38:11	17 A Well, that's a better question for a technical 13:40:25
18 Q Do you agree that the majority of doctors have a 13:38:15	18 expert, but my understanding is they could not if there 13:40:27
19 preference between tumor informed versus tumor-agnostic 13:38:17	19 was no tissue to study. 13:40:29
20 MRD tests? 13:38:23	20 Q So in that situation, a tumor-informed MRD test 13:40:33
21 A To the extent tissue is available, I think the 13:38:24	21 is not an acceptable alternative to a tumor-naïve MRD 13:40:36
22 research shows that all else being equal, doctors 13:38:28	22 test? 13:40:43
23 currently have expressed a preference for tumor 13:38:31	23 A Again, I would defer to the technical or 13:40:43
24 informed, that's true. 13:38:35	24 physician experts, but my understanding if there is no 13:40:44
Page 62	Page 64
1 Q So it's not your opinion that doctors don't -- 13:38:36	1 tissue to sample, you can't have a tissue-informed test. 13:40:48
2 There is a double negative in that, so let me ask a 13:38:38	2 Q If a tissue sample is available, what are the 13:40:55
3 different question. 13:38:41	3 reasons that an oncologist would order a tumor-naïve MRD 13:40:57
4 Is it your opinion that doctors care whether an 13:38:42	4 test instead of a tumor-informed MRD test? 13:41:03
5 MRD test is tumor informed or tumor naïve? 13:38:45	5 MR. NOLAN: Objection, sorry. 13:41:07
6 A I don't know how to answer what they care about. 13:38:51	6 THE WITNESS: Again, I think that's a better 13:41:08
7 My understanding is that's not the first criteria they 13:38:53	7 question for the technical or physician experts, but I 13:41:10
8 use to select a test. But if you ask them in a survey 13:38:57	8 do understand that there may be benefits that are argued 13:41:12
9 between informed and naïve what's your preference, that 13:39:01	9 by Guardant with respect to false negatives and false 13:41:18
10 all else being equal, they have historically expressed a 13:39:05	10 positives for a given indication, or there may be 13:41:23
11 preference for tumor informed. 13:39:09	11 restrictions on what indications are approved. 13:41:26
12 Q What advantages does a tumor-informed MRD test 13:39:12	12 So it would be a physician-based decision, and 13:41:31
13 have relative to tumor naïve? 13:39:16	13 I don't have an opinion as to generally how those are 13:41:34
14 A I think that's more of a clinical question that 13:39:18	14 classified. 13:41:37
15 I would defer to the experts, technical experts. 13:39:20	15 MR. SMITH: Q Can you take a look at your 13:41:39
16 Q All right. But you agree that tumor-informed 13:39:23	16 declaration, paragraph 36, I think. I'll ask you a few 13:41:55
17 and tumor-naïve approaches each have their own benefits? 13:39:25	17 questions about that. 13:41:58
18 A My understanding of the record is that there are 13:39:29	18 Are you there? 13:42:05
19 unique roles within the market for each and that there 13:39:32	19 A I am. 13:42:06
20 is, for example, evidence from physicians describing 13:39:35	20 Q Okay. In this paragraph you quote a portion of 13:42:06
21 that, that there is a role for both within the market. 13:39:38	21 Mr. Sikri's declaration where he states, "Tumor-informed 13:42:10
22 Q And oncologists are aware of the benefits and 13:39:41	22 and tumor-naïve MRD assays provide healthcare 13:42:15
23 drawbacks of tumor-informed and tumor-naïve approaches? 13:39:43	23 professionals with the flexibility necessary to make 13:42:18
24 MR. NOLAN: Objection. 13:39:47	24 informed treatment decisions." 13:42:21
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IN THE UNITED STATES DISTRICT COURT
FOR THE MIDDLE DISTRICT OF NORTH CAROLINA

NATERA, INC.,) CASE NO. 1:23CV629
Plaintiff,)
)
vs.)
)
NEOGENOMICS LABORATORIES, INC.,) Greensboro, North Carolina
Defendant.) November 9, 2023
2:02 p.m

TRANSCRIPT OF THE **TUTORIAL HEARING**
BEFORE THE HONORABLE CATHERINE C. EAGLES
UNITED STATES DISTRICT JUDGE

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Proceedings recorded by mechanical stenotype reporter.
Transcript produced by computer-aided transcription.

Natera v NeoGenomics -- Tutorial Hearing -- 11/9/23

P R O C E E D I N G S

THE COURT: Good afternoon. We're here in Natera against NeoGenomics, 23CV629, for you all to educate me a bit on the science involved.

I read all the briefing on the motion for preliminary injunction and skimmed the briefing on the Defendant's motion to dismiss, which I think may not be complete. I can't remember exactly. But I've read some more stuff about the section -- the patentability argument I'll just say. But I would be glad to have you all explain this to me better.

I did just yesterday see an article -- I think it was in the *Guardian* -- about some research in London for an at-home blood test they're trying to develop for brain cancer, and it sounded a lot like this; but it might not have been exactly the same. Obviously that was not scientific literature, but I think it falls into the category of once you know a little something about it, it starts showing up everywhere in your life. So there it was.

Okay. So I hope you all have agreed on a plan for your use of your time this afternoon. I have until 5:00, and we will need to take a break if we use all of that time because I can't sit that long without a break.

Have you all conferred about how you want to go forward? No. Nobody is nodding "yes" or "no."

(Indiscernible cross-talk.)

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1 **THE COURT:** I'm sorry?

2 **MS. HABERNY:** Your Honor, we assumed approximately 30
3 minutes but additional time for questions per side, but there
4 was no formal stipulation or anything.

5 **THE COURT:** That sounds great.

6 Well, does Plaintiff want to go first?

7 **MR. VAN ARNAM:** Just a couple new faces you haven't
8 seen. Rob Van Arnam with Williams Mullen, Your Honor.

9 **THE COURT:** Oh, yes, and I'm not sure if the court
10 reporter has been with us so everybody introduce themselves.

11 Thank you.

12 **MR. VAN ARNAM:** With me is Dr. Haberny -- we got a
13 lot of doctors here -- and Dr. Srinivasan -- and I can get you
14 the spellings if it's not on the record -- Dr. Michael Metzker
15 who will be presenting the tutorial today, and in-house counsel
16 for Natera, Dr. Sean Boyle.

17 **THE COURT:** All right. Great. Thank you.

18 **MR. MORROW:** John Morrow, Your Honor. Pleased to be
19 here.

20 **MS. RYAN:** Liz Ryan. Unfortunately, no doctors on
21 this side.

22 **THE COURT:** And I know Mr. Reines is -- well, he's
23 observing by video, which I was glad to allow him to do.

24 Okay. Go ahead then for the Plaintiff.

25 **MS. HABERNY:** Thank you, Your Honor. We do have a

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1 slide deck. I believe we have handed up a copy to Your Honor.

2 **THE COURT:** Is that this?

3 **MS. HABERNY:** It's the other one.

4 **THE COURT:** The other one. This one?

5 **MS. HABERNY:** Yes, Your Honor.

6 And we also have a live PowerPoint that we would like
7 to use for the presentation. If we can have that turned on?

8 Okay. So I would like to start with a brief
9 introduction to the Plaintiff, Natera. So Natera is the
10 Plaintiff in this case. I would like to briefly introduce
11 Natera and the genesis for the technology that's underlying the
12 patents that are at issue in this proceeding.

13 So Natera was founded in 2004 to focus on noninvasive
14 methods for analyzing genetic conditions. And Natera has,
15 since, then developed a large number of products, as Your Honor
16 can see here in a year-by-year timeline of product development,
17 and all of these products are intended to test for and address
18 genetic conditions. So many of them stem from the same basic
19 concept which is analyzing mixed samples of cell-free DNA, and
20 those samples can come from a number of different sources and
21 be used to analyze a number of genetic conditions. But all of
22 them arise from the same concept of analyzing cell-free DNA
23 that's circulating in the blood and looking for types of
24 cell-free DNA that are very low abundance in the blood.

25 So the first cell-free DNA product that Natera

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1 developed was called Panorama™. You can see here in 2013 that
2 product was launched. It's a cell-free DNA test for
3 noninvasive prenatal care, and it looks for fetal abnormalities
4 using the blood from a pregnant woman.

5 And using this similar technology, Natera launched
6 its oncology product which is called Signatera™ in 2017. And
7 that Signatera™ product looks for tumor-derived cell-free DNA
8 in the blood of a cancer patient.

9 Then using similar technologies, in 2019, Natera
10 launched another cell-free DNA product that's intended to test
11 for organ transplant rejection or organ transplant monitoring,
12 and that one, similarly, looks for cell-free DNA derived from
13 an organ donor in the blood of the organ transplant recipient.

14 So all of these tests are looking for minute amounts
15 of cell-free DNA circulating in the blood.

16 Can we have the next slide, please.

17 So I would like to focus for a moment on the oncology
18 testing which is at issue in this case. And what these -- what
19 this technology does is it monitors for relapse or recurrence
20 of cancer. So a person can have cancer and then be treated and
21 go into remission; in which case, almost no or very, very small
22 amounts of tumor-derived cell-free DNA will be in the cancer
23 patient's blood. But if the cancer patient goes into relapse
24 or if the cancer starts coming back, then you will begin to
25 detect tumor-derived cell-free DNA in the blood.

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1 And so the standard approaches to identify cancer
2 relapse involve x-rays, CT scans, biopsies, using antibodies.
3 These are techniques that are either invasive or not very
4 sensitive. So what Natera has done is developed ways to get a
5 more sensitive test that does not involve invasive procedures,
6 and there are other companies doing similar things.

7 And there are two basic approaches to monitoring
8 relapse. One is using what's called a nonpersonalized MRD, or
9 molecular residual disease, test. And companies like Guardant
10 Health and Personal Genomics use basically an off-the-shelf
11 test looking for -- every patient gets the same test, and they
12 look for the same mutations -- same cancer-related mutations in
13 all patients.

14 And then there is a personalized approach to testing
15 for minimal residual disease, which is the condition that
16 occurs when cancer relapse happens. And Natera and NeoGenomics
17 offer tests in the personalized MRD testing phase, and what
18 these do is they identify mutations that are unique to a
19 particular patient, and then they specially design a test only
20 to look for those mutations that are unique to the patient.

21 So the patents that are being asserted in this case
22 relate to this personalized minimal residual disease testing,
23 and there are two patents asserted in this case: One I will
24 refer to as the '454 patent and the other as the '035 patent.

25 So we brought our expert Dr. Michael Metzker here to

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1 explain the basis of the technology, but, quickly, I will just
2 go through the claims and some of the terms that we would like
3 him to address for Your Honor so that you can hear them and
4 have them in mind.

5 The '035 is a method for amplifying and sequencing
6 DNA. And Dr. Metzker is going to talk about the elements that
7 are highlighted, including tagging isolated cell-free DNA with
8 one or more universal tail adaptors. That's to generate tagged
9 products.

10 And amplifying the tagged products one or more times
11 where the amplification steps comprise targeted amplification
12 of a plurality of what are called single-nucleotide polymorphic
13 loci, and this is done in a single reaction volume. And the
14 amplifying steps can introduce a barcode of one or more
15 sequence tags.

16 And then sequencing, the plurality of SNP loci --
17 that's a lot of big words, I know -- on the cell-free DNA with
18 massively parallel sequencing.

19 So our expert is going to go into each of those in
20 detail.

21 Can we have the next slide.

22 Then we have a dependent claim --

23 **THE COURT:** I mean, we're not here for claim
24 construction.

25 **MS. HABERNY:** We're not.

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1 **THE COURT:** I don't want to talk about claim
2 construction. We're just here for you all to explain these
3 words to me and the background and that kind of thing.

4 **MS. HABERNY:** Absolutely, Your Honor.

5 And so we have a dependent claim which recites the
6 universal tail adaptors comprising a first universal tail
7 adaptor and a second universal tail adaptor, so using two
8 universal tail adaptors.

9 And then Dependent Claim 13, further comprising
10 amplifying with a first primer comprising the first tail
11 adaptor; second primer comprising the second universal tail
12 adaptor.

13 Can we go to the second slide.

14 I will just briefly overview the concepts underlying
15 the '454. So this is a method for preparing a plasma sample.
16 It's useful for detecting one or more single-nucleotide variant
17 mutations in the sample. It involves several steps. One of
18 them is performing whole exome sequencing or whole genome
19 sequencing on a tumor sample, and that's to identify
20 tumor-specific single-nucleotide variant mutations.

21 And then performing targeted multiplex amplification,
22 and that's to amplify in multiplex form 10 to 500 target loci
23 encompassing different tumor-specific single-nucleotide variant
24 mutations. It's done now on cell-free DNA that's isolated from
25 a plasma sample rather than a tissue sample, and that's to

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1 obtain amplicons having this certain length of 50 to 150 bases.
2 That amplification, like the amplification in the '035 patent,
3 happens together in the same reaction volume. So you're
4 amplifying multiple things together in the same reaction
5 volume.

6 And then sequencing the amplicons to obtain sequence
7 reads and, thereafter, detecting one or more of the
8 tumor-specific mutations that are present and doing this with a
9 sequencing depth of read of at least 50,000 per target locus.

10 So big mouthful.

11 **THE COURT:** Yeah. I don't understand any of it.
12 That's why we're here. So, I mean, I can read it.

13 **MS. HABERNY:** Exactly. Well, Your Honor, with that,
14 I will just quickly, just to frame it, provide --

15 **THE COURT:** I mean, I learned some about it in
16 reading y'all's briefs. It's not true that I don't understand
17 anything about it. I did read your briefs. I do understand
18 some things about it, but not much.

19 **MS. HABERNY:** Okay. Understood, Your Honor.

20 If we could go to the other dependent claims.

21 **THE COURT:** I mean, what I am trying to say is it's
22 not very helpful to me for you to read this. I actually
23 already read the claims that are at issue.

24 **MS. HABERNY:** Okay. Then I will turn it over now to
25 Dr. Metzker. He has a Ph.D. in molecular human genetics.

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1 **THE COURT:** I read his declaration.

2 **MR. VAN ARNAM:** Your Honor, would you prefer that he
3 do it from --

4 **THE COURT:** Well, he's not a witness. I mean, I am
5 not going to take this as evidence. He can do it from right
6 there.

7 **DR. METZKER:** Good afternoon, Your Honor.

8 **THE COURT:** You do have to speak into a microphone.

9 **DR. METZKER:** I've got one right here. I hope that
10 is loud enough.

11 So if we can go to the next slide.

12 I will try to do this very slowly. And if you have
13 questions, please feel free to ask them.

14 So we're talking about DNA. DNA is a molecule that
15 exists in cells. It's in the center of the cell, as I'm
16 showing here in the nucleus. It's organized as chromosomes.
17 And what I am showing on the right-hand side is DNA being
18 unwound into what we all know as a double helix. And I just
19 show the molecule on the right-hand side. It's a histone, and
20 it's DNA wrapping around a histone. And that's just part of
21 the way the chromosome is organized within the cell.

22 If we can go to the next slide.

23 So as I mentioned, DNA is a double helix.

24 **THE COURT:** I actually do understand about the four,
25 CTGA.

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1 DR. METZKER: Perfect. Excellent.

2 THE COURT: Okay. I'm with you so far.

3 DR. METZKER: So let's go to the next slide then.

4 And what's special about the A, C, G, and T is this
5 rule in biology called base pairing. So we refer to ACGT as
6 bases, and A always pairs with T, and C always pairs with G.
7 And that's important because when you separate those strains,
8 the information in one strain can be used to copy a new strand
9 to make a new double-stranded molecule. We use that strand
10 information also to determine sequence information from the
11 actual DNA molecule itself.

12 If we can go to the next slide.

13 So we're here to talk about what is cell-free DNA,
14 because I've just told you DNA is found in cells and in the
15 nucleus. But in normal health, cells die; and when they die,
16 their membranes break open and all the materials within the
17 cell are extruded into the blood. And I am trying to
18 illustrate that here. And part of what gets extruded into the
19 blood is that genomic DNA. We call that genomic DNA, which was
20 in the cell and now goes into the bloodstream.

21 Now, genomic DNA is a very large molecule. Our
22 genome is made up of 3 billion As, Cs, Gs, and Ts. The
23 cell-free DNA we're talking about here is small. It's about
24 140 to 170 letters, or we call those base pairs. And it is
25 because of that histone where the DNA wraps around it and

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1 protects it when the DNA is extruded into the blood, because we
2 have scissors in our blood that are trying to remove DNA and
3 clear it and go through our kidney system.

4 So in the next slide, most of the cell-free DNA
5 that's found in our bloodstream come from normal cells. Normal
6 cells also live and die, and the way the body gets rid of dead
7 cells is they break up their membranes and they extrude all of
8 their inners into the bloodstream, and it gets filtered out.

9 But in the case of cancer, cancer cells also live and
10 die. In fact, cancer cells grow quite rapidly forming tumors,
11 but they do also undergo cell death. And the genomes in a
12 tumor cell, the DNA in a tumor cell, is different from the DNA
13 in a healthy cell. The DNA in a tumor cell starts accumulating
14 what we call mutations, or changes, in the As, Cs, Gs, and Ts
15 of our genome. So the tumor cells also undergo cell death.
16 And cell-free DNA goes into the bloodstream as well.

17 So in a cancer patient, when you take blood and you
18 isolate cell-free DNA from the blood, it is a mixture of
19 healthy cell-free DNA and what we call ctDNA, circulating tumor
20 DNA. And these circulating tumor DNA molecules are highly
21 specific biomarkers for cancer detection.

22 And the challenge of using cell-free DNA is the ctDNA
23 is in very low abundance. It's basically trying to find the
24 needle in the haystack.

25 And I will try to go through some more to help you

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1 understand what that means.

2 **THE COURT:** It means you have to amplify it.

3 **DR. METZKER:** That's part of it, yes.

4 **THE COURT:** Okay. Go ahead.

5 **DR. METZKER:** Can we go to the next slide.

6 So, here, we heard that there's normal DNA and
7 there's tumor DNA. And the way these personalized assays work
8 is a tumor sample is first obtained from an individual -- and
9 we're doing a biopsy here from a breast cancer patient -- and
10 blood is isolated. We analyze the cell fraction of the tumor
11 first to identify which mutations are in the sample, and we do
12 that by comparing the DNA from the tumor sample with DNA from
13 normal cells, because there is a lot of -- we call it
14 variation, changes in our genomes from individual to
15 individual. And the point of this test is to find the
16 mutations that are specific to the tumor.

17 **THE COURT:** Right, because we all have all kinds of
18 mutations that --

19 **DR. METZKER:** That's right. That's exactly right.

20 So what I am showing you here are now two DNA
21 sequences where we are now comparing tumor DNA and normal DNA.
22 In cancer, we call those somatic mutations. These are changes
23 that happen after we're born, and we accumulate these
24 throughout our lifetime.

25 **THE COURT:** Somatic?

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1 **DR. METZKER:** S-O-M-A-T-I-C.

2 And what we're doing here is now identifying by
3 comparing normal DNA and tumor DNA to find the differences,
4 those changes that are associated with the cancer cell. And
5 those are important biomarkers because personalized assays are
6 then developed specifically for these variants that can then be
7 tested in the blood liquid biopsies, where you take a blood
8 sample and you do the test to try to identify the presence or
9 absence of ctDNA.

10 So if we can go to the next slide.

11 As I mentioned, a tumor sample is identified on the
12 left-hand side. In the middle is where these variants have
13 been identified. A personalized test for each patient has been
14 designed to target what we call SNPs or SNVs. These are
15 single-nucleotide polymorphisms -- can we actually go back one
16 slide.

17 I failed to mention, on this slide, Slide 19,
18 highlighted in yellow would be an example of a
19 single-nucleotide polymorphism. It's one of the terms in the
20 claim. We call it an SNP. And it's where at one location, or
21 one locus, in our genomes the sequence differs by one
22 nucleotide. That's where single-nucleotide polymorphism comes
23 from, or sometimes it's referred to as a single-nucleotide
24 variant, or an SNV. And that's very important just to
25 understand what that term means.

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1 **THE COURT:** Okay.

2 **DR. METZKER:** So we can go forward now.

3 So we've now designed our assays in the middle for
4 those SNPs and those SNVs. And now on the right-hand side is a
5 timeline of what could happen for the course of a patient where
6 prior to treatment, which is that green rectangular box, a
7 blood sample is now drawn and ctDNA is detected. And in this
8 example, it's pretty high up on the y-axis. Treatment is now
9 given. Another blood test is tested with a personalized assay,
10 and the amount of ctDNA can be measured, and you can see it
11 drops. Hopefully, the outcome of this patient is you never
12 detect ctDNA again. And that is a biomarker for the cancer
13 coming back. The cancer patient relapses over some period of
14 time. And these tests are powerful because the ctDNA can be
15 detected months before cancer is detected by traditional
16 radiographic imaging or CT scanning or things like that.

17 So if we can go to the next slide.

18 So now I am going to go to some basic molecular
19 biology. This is amplification. In our field it's basically
20 copying DNA, making lots of copies. And the buzzword that we
21 use a lot and in the example I am showing here is called
22 "polymerase chain reaction," or PCR. And the PCR cycle and the
23 PCR method is well known in the field. It's been around since
24 the 1980s. And it really is a test tube full of some reagents
25 that -- you basically change the temperatures; you go up and

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1 you go down, and you manipulate the DNA to make multiple copies
2 of itself.

3 So if we can start the video.

4 The first step is to separate our double-stranded
5 DNA. So this is cell-free DNA. We have now separated the
6 double strands into single strands. You can see all the base
7 pairs there. Again, I mentioned the base pairs are really
8 important because I can add small little pieces of DNA,
9 synthetic -- you can make them in the laboratory -- called
10 primers. These primers will hybridize -- if we continue with
11 the animation -- hybridize, or bind, specifically to the region
12 where we have identified the SNP. Because of those
13 base-pairing rules, we can tell the primers exactly where to go
14 to hybridize on both of the single strands on opposite sides.

15 So as illustrated here, on the top strand the primers
16 hybridized on the right bottom part of the template, or the DNA
17 molecule. And on the bottom strand, the primer has hybridized,
18 or has bound, to the left-hand side of the bottom strand.

19 This is important because in this mixture -- and we
20 just do this by heating the mixture up, because DNA is not
21 stable when it's hot, and then we cool it back down, and these
22 primers can now bind to the regions of interest.

23 And then there is also an enzyme in there called a
24 polymerase that copies the DNA strand. Because of the
25 base-pairing rules, it can make a mirror image of both strands

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1 based on what information is in the existing strand. So now
2 we've gone from one double-stranded DNA molecule to two
3 double-stranded DNA molecules. We've done our first cycle of
4 PCR. Now, this is in a test tube, and it's all automated.

5 If we can go to the next slide.

6 We'll go -- there's -- we'll go into more cycles.
7 The PCR method usually involves more than one cycle. Sometimes
8 it involves as many as 30 cycles of separating the strands,
9 binding the primers, extending with the polymerase, and making
10 two copies into four copies, four copies into eight copies.
11 And over 25 cycles, you've created more than a million copies
12 now in a relatively short period of time.

13 The claim language is up, but I'm actually going to
14 just explain the science behind what is amplifying, which is
15 what we just talked about, and sequencing DNA.

16 So if we can first go to the first claim element:
17 Tagging isolated cell-free DNA to generate tagged products
18 using isolated cell-free DNA from a blood sample.

19 So in the next slide, I'm first illustrating how you
20 isolate circulating DNA from a blood sample. And on the left,
21 under No. 1, you would go into your doctor's office, and she or
22 he would draw blood. And then it's sent to the laboratory, and
23 the laboratory technicians can put it into a centrifuge, which
24 can spin and separate the blood into separate layers. And so
25 the top layer is the plasma layer, which does not have cells.

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1 Because of the density between white blood cells and red blood
2 cells, you can actually separate white blood cells, which is
3 that middle fraction. And then on the bottom, red blood cells
4 are the heaviest. They're at the bottom. You take the top
5 layer, the plasma sample, and you can isolate cell-free DNA
6 from that plasma sample. And that's what's shown in the last
7 part of that slide.

8 Now, I'll just throw this out. The white blood cells
9 are also used in this first step because it's the normal DNA
10 that you can then compare to your tumor DNA to identify those
11 variants.

12 If we can go to the next slide.

13 So the claim requires tagging. In our field that
14 means putting other DNA sequences on the end of our cell-free
15 DNA molecule, and there are at least two ways to do this. And
16 the first way I'm showing is a method called ligation.
17 Ligation is simply gluing -- physically or chemically gluing
18 together these small little DNA molecules to our circulating
19 cell-free DNA. And I illustrate it here by using glue, but
20 there are, as you can imagine, other enzymes that are used to
21 do this gluing process.

22 **THE COURT:** And what are you gluing it to?

23 **DR. METZKER:** The cell-free DNA. So we are tagging
24 cell-free DNA. So the middle double-stranded molecule is our
25 cell-free DNA --

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1 **THE COURT:** Yes.

2 **DR. METZKER:** -- that we just isolated from the
3 plasma.

4 **THE COURT:** And what's on the end that you're gluing
5 to it?

6 **DR. METZKER:** Those are our tags. We call those
7 tags.

8 **THE COURT:** And what are the tags?

9 **DR. METZKER:** They are more DNA molecules, but they
10 have -- they're synthetic. We designed them in the laboratory,
11 and they are important for the downstream process of either
12 amplifying or sequencing.

13 And if we can go to the next slide.

14 The tagging can also be done through amplification.
15 So we've gone through this a bit. So if we can advance the
16 slide. We separate the strands. We have our little primers
17 that bind specifically to the regions that we want to identify
18 the single-nucleotide polymorphism. But on your primers we can
19 add additional sequences as well. We can add tags to the ends
20 of the primer. And by doing that, when the primer that's now
21 tagged binds to the cell-free DNA and then makes a new copy as
22 a double-stranded -- and if we can go to the end -- we've now
23 tagged the cell-free DNA in the first cycle of PCR, because the
24 primer has a tag that's now attached and now part of the
25 cell-free DNA molecule.

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1 So those are two common ways of tagging DNA
2 molecules.

3 (Pause in the proceedings.)

4 **DR. METZKER:** And I see you're taking notes.

5 **THE COURT:** Thank you.

6 **DR. METZKER:** You're welcome.

7 So if we can go to the next slide.

8 Now, the next step is amplifying the tagged
9 product -- so we just made a tagged product -- and one or more
10 times to generate a final amplification. And this has two
11 amplification steps wherein one of the amplification steps
12 comprises targeted amplification of the plurality of
13 single-nucleotide polymorphisms. We went over that. But it's
14 all done in one tube. So if I have identified in my cancer
15 patient, let's say, ten different SNPs that we think are
16 related to the tumor, this claim now requires that I am
17 amplifying all ten of those simultaneously in the same tube.

18 So if we can go to the next slide.

19 So I mentioned we'd continue with how the PCR cycle
20 works. In our first cycle, we've made two molecules from one.
21 They're both tagged. I would then heat the reaction up, and
22 the DNA molecules separate again into single strands. I have
23 my primers with their tags on them, and they now bind to the
24 tagged product, and now I'm amplifying the tagged product in
25 this PCR cycle. So now I've gone from two molecules at the top

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1 of the slide, and I now have four molecules at the bottom of
2 the slide. And in PCR everything multiplies by two with each
3 cycle. So the more cycles, the more copies that are made.

4 If we can go to the next slide.

5 And so this is just illustrating -- if we can just
6 continue to go -- the second cycle, I've made four, and the
7 third cycle, I've made eight. And by 30 cycles, I've made a
8 million or more tagged products in this amplification.

9 Now, this also -- because we're doing a plurality of
10 SNPs, I have to not only do 30 cycles, but I have multiple
11 primers that look at different regions across my genomic DNA.
12 And so what I'm trying to illustrate here are three target
13 locus positions. These are just three places in our genome.
14 Think of -- we've identified our first SNP on chromosome 1;
15 we've identified our second SNP on chromosome 5, and the third
16 one on chromosome 10. And in one test tube I want to apply
17 this PRC method to target all three of those simultaneously.
18 We call that multiplex PCR.

19 And what I'm illustrating now is under each locus is
20 a cell-free DNA molecule where I've identified --

21 **THE COURT:** A cell what?

22 **DR. METZKER:** Cell-free DNA molecule.

23 **THE COURT:** I thought you said self-redeeming, and I
24 was very confused.

25 **DR. METZKER:** I hope not.

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1 **THE COURT:** Okay. Go ahead.

2 **DR. METZKER:** -- three cell-free DNA molecules, and I
3 have boxes where I have found a SNP that is associated with the
4 patient's tumor. And now I am going to amplify those three
5 different cell-free DNA molecules because I am interested only
6 in those that I have identified when I did my first test of
7 identifying the SNPs. And because of that amplification, now
8 you end up with a tube full of lots of copies of those three
9 different loci all having SNPs that we're interested in.

10 Now, I'm showing you an example of three, but you can
11 actually multiplex to 10, 20, or even 100 different SNP loci in
12 a single reaction.

13 If we can go next to the next slide.

14 And then there's another step in this amplifying step
15 that introduces a barcode in one or more sequencing tags.

16 If we can go to the next slide.

17 And we can do that, again, through the PCR step by
18 adding on different sequences to our primers. Our primers are
19 our guide to the genome where if we're interested in Locus 1,
20 our primers take us right there. They have specific sequences
21 because of the base-pairing rules.

22 But on the ends of those primers, we can add
23 additional sequences in -- on the left-hand side on the top --
24 on my primer that has a blue arrow on the left-hand side, I'm
25 adding a sequencing tag. And I'll explain what that means in

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1 just a minute. But I'm just illustrating how that's done.

2 And then on the right-hand side under my -- or at my
3 primer that has the red arrow, on the right-hand side of that,
4 I can introduce what is called a barcode. And a barcode, if we
5 go to the next slide, is essentially the same thing you use
6 when you go to the grocery store and you're checking out, and
7 they scan and they know that your can of chicken soup is \$1.25.
8 But the way we use bars in molecular biology is it's, again,
9 based on DNA sequence. So we can code -- we can give that
10 particular sequence an identifier. We can say, okay, Code 1 is
11 going to be AACG, and Code 2 will be TTCA, and Code 3 will be
12 AGAG. So we know the sequence; and when we can read the
13 sequence, we know what the barcode is. And that's important
14 because then we can identify which patient the sequencing data
15 is coming from.

16 The other thing that I mentioned are sequence tags.
17 So we're going to go through what massively parallel sequencing
18 is, sometimes referred to as next generation sequencing. There
19 are several companies that make sequencers that we all use in
20 life sciences. The one I am going to give an example of is
21 called Illumina, and Illumina is one of the leading
22 manufacturers of massively parallel sequencing. They have a
23 very specific protocol that you have to follow if you are to
24 run their instrument. You have to put on what they call
25 sequencing tags. These are tags that are very specific to

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1 their platform; and if you add the sequence tags, you can run
2 your sample on their platform and get useful data. If you do
3 not put sequencing tags on the ends of these DNA fragments,
4 their platform doesn't work. It is an absolute requirement for
5 massively parallel sequencing to have these sequence tags that
6 are specific to the sequencing platform that you're using.

7 So that's what a sequence -- that's why we have a
8 sequence tag.

9 **THE COURT:** Okay.

10 **DR. METZKER:** Go to the next slide.

11 So the last step is now sequencing, and I'll talk
12 about what that is. A plurality of SNP loci, those are our
13 single-nucleotide polymorphisms, and the loci is just the
14 position on the genome that we've identified that's associated
15 with this patient's tumor cells.

16 Conducting massively parallel sequencing on the final
17 amplification product where the SNPs comprise anywhere from 25
18 to 2000 loci -- okay. So I gave an example of three different
19 loci in demonstrating multiplex. To read on this claim, you
20 would have to do at least 25 and you can do up to 2,000 in a
21 single reaction tube, all having primers that target specific
22 regions and are doing the copying, the amplification step,
23 simultaneously.

24 If we go to the next slide.

25 So this is a real live picture of one of the Illumina

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1 sequencers that is used in our field, and it shows here the
2 amplification product on the left and then there is an arrow
3 that says "Insert into machine." Well, the only way you can
4 actually do that is you have to put on these sequencing tags on
5 the ends of these amplification products, and there are some
6 other steps that are involved before they go onto the
7 sequencer.

8 Now, the sequencer is a machine that, what we call,
9 reads DNA. It actually takes the single-stranded amplified
10 product which has all the sequence tags and the barcodes, and
11 it uses, again, a polymerase, because the polymerase can read
12 one strand. It can read I have an A here; I have a T here; I
13 have a C here. And it's starting to incorporate, or add,
14 nucleotides to make double-stranded DNA. So the sequencer not
15 only replicates DNA to make double-stranded DNA, in the process
16 of doing that, it can individually read which base is there,
17 and it determines what the order of the letters are in the DNA
18 sample. And we call that a sequence read or reading the
19 sequence.

20 And that machine there then spits out information
21 into a computer file that's represented on the right-hand side,
22 and now it's showing you what the sequence is for each
23 cell-free DNA molecule that we amplified, we tagged, and we
24 added sequence tags and barcodes.

25 Now, these are called massively parallel sequencing,

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1 because these sequencers can generate up to a billion or more
2 sequence reads in one run from a sample that came from that
3 test tube. So we're looking at billions of sequence reads that
4 have all read the cell-free DNA that was in our original
5 sample.

6 All right. We are just going to briefly talk about
7 some of the terms in '454 just from a technology point of view.

8 So the claim is preparing -- a method of preparing a
9 plasma sample from a subject having cancer or suspected of
10 having cancer for detecting one or more single-nucleotide
11 variants. So a single-nucleotide variant and a single
12 nucleotide polymorphism are pretty much the same thing. There
13 are differences between the normal DNA and the cancer DNA at
14 one position, at one letter. That's why we call them single
15 nucleotide. Variant is it's just different. Polymorphism has
16 a little bit of a genetic understanding. But for purposes
17 here, they are pretty much the same. And this is isolated from
18 a plasma sample.

19 So if we can go to the next slide.

20 The first step of this -- that was the preamble -- is
21 performing whole exome sequencing or whole genome sequencing of
22 a tumor sample of a subject, and the purpose is to identify
23 these tumor-specific SNVs. That's a lot right there.

24 So if we go to the next slide, whole exome -- I will
25 try to simplify this. So I'll do whole genome first. A whole

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1 genome just means you're just going to read all the DNA that
2 used to be in your cell or -- yeah, that is in your cell,
3 because we're doing tissue samples here. So you isolate cells;
4 you extract the DNA from the tumor cell, and then you just
5 sequence the entire genome.

6 And I told you the genome is big. It's like three
7 billion letters. That's called whole genome sequencing, and
8 that's a way of trying to identify where these tumor specific
9 mutations exist throughout our whole genome. This is how the
10 personalized test is built.

11 **THE COURT:** Because you have to have something to
12 compare it to?

13 **DR. METZKER:** Well, you do, but each patient is
14 different. These are personalized tests. We don't know where
15 things are in our genomes for any given cancer patient. So you
16 do that by sequencing their whole genome, and then you compare
17 their tumor genome to their normal genome. And through that
18 same exercise I showed before, you look for where their
19 differences are. And then you can say, okay, on my blood test,
20 my liquid biopsy, I'm going to focus on those changes that I
21 think are associated with my tumor.

22 Now, the second is whole exome sequencing. Our --
23 I'm sure you've heard we have genes. And genes make proteins.
24 You know, they make our hair color and our eyes and all of
25 that. Genes in scientific terms are called exomes. They are

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1 made of exomes. And the genome is organized in a way that our
2 genes only constitute like 1 or 2 percent of your whole genome.

3 So whole exome sequencing really only focuses on DNA
4 sequences that are responsible for making the proteins in the
5 body, what we call the coding region that makes a gene into
6 some functional product that works in each cell. So it's kind
7 of a shorthand way of saying I'm only interested in those
8 mutations that affect the way a protein works in my body.
9 That's whole exome sequencing. So the claim can do one or the
10 other.

11 On the right-hand side, again, the purpose is to find
12 these single-nucleotide variants that are specific to that
13 patient's cancer. And that's where we then build a
14 personalized test.

15 So that's what those terms mean.

16 If we can go to the next slide.

17 So the second step of this claim is performing
18 targeted multiplex PCR -- and we've talked about what targeted
19 multiplex PCR is, but I'll go over that briefly again to the
20 point that Your Honor understands at least in concept what is
21 happening in the test tube -- amplifying 10 to 500 target loci
22 from cell-free DNA isolated from a plasma sample from a
23 subject.

24 If we can go to the next slide.

25 So, again, this is just the steps of isolating

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1 cell-free DNA from a plasma sample. We have gone through that.

2 And the next slide.

3 This is the amplification step we've talked about
4 before whether the strands are separated because we heat it up.
5 Then we have our primers in the mix, and they bind and then we
6 can use our enzyme and we can make two copies of
7 double-stranded DNA from one copy, and the second cycle goes to
8 four, the third cycle goes to eight, and now over 30 cycles, we
9 have more than a million amplified molecules in our reaction.

10 **THE COURT:** Right.

11 **DR. METZKER:** And, again, we've done this by
12 multiplex. So not only do we have a top primer and a bottom
13 primer, but we have those same pairs by 10, 20, 50 different
14 SNP loci that we're trying to target in our reaction on those
15 that we've identified from whole genome sequencing or whole
16 exome sequencing. That's the starting point of how we -- the
17 test to build these primer steps to amplify the cell-free DNA
18 comes from.

19 The next slide.

20 So the goal is to obtain amplicons having a length of
21 50 to 150 bases, and that's all based on where -- and we do
22 this in the laboratory. We design what those sequences are
23 that make a primer where they bind to the genome. Where the
24 top primer and the bottom primer hybridize, that is the
25 goalpost of how big that amplicon will be.

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1 Now, that's important because you can use that as a
2 way of defining what the length is, and then, as I mentioned
3 before, we can add additional sequences on the ends such as
4 barcodes. And I know one of the dependent claims is a
5 barcoding step, and that's how that would be introduced in this
6 reaction.

7 If we can go on to the next slide.

8 And now we're going to sequence amplicons. And it
9 kind of sounds like the last step of the '035, but there is
10 actually an important difference, and I want to highlight that.

11 Sorry. So if you'll just go back.

12 And where I want to really focus you is where the
13 sequencing has a read depth of at least 50,000 per target loci.
14 So if we go to the next slide, again, this is massively
15 parallel sequencing. I've tried to explain that it can produce
16 as many as a billion sequence reads from a single sample that's
17 been fed into the machine. If you look towards the middle of
18 the slide, there's a histogram, and on the x-axis, those
19 numbers are the numbers --

20 **THE COURT:** The bar chart?

21 **DR. METZKER:** Yes, in the chart.

22 **THE COURT:** Okay.

23 **DR. METZKER:** So between the machine and the --

24 **THE COURT:** Yeah, I see it. I just didn't know what
25 a histogram was, but I know what a bar chart is.

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1 **DR. METZKER:** Bar chart.

2 **THE COURT:** Okay. You can call it a histogram now
3 because now I know what it means.

4 **DR. METZKER:** Very good.

5 Okay. And this is to illustrate the depth of read,
6 and I will try to explain what that means. But, first, what
7 it's just showing you is that on the x-axis it goes anywhere
8 from 50,000 to, it looks like, 340,000. The screen is a little
9 blurry, but this is for one SNV loci in our test. Remember,
10 we're doing 10, 20, 30 different loci in our multiplex targeted
11 amplification step, but now we're reading -- we're reading the
12 DNA. On that particular SNP, we read that 50,000 times. So we
13 generate enough reads -- sequence reads. That's called a read
14 depth. If you read that SNP 100,000 times, you would say you
15 have a read depth of 100,000 times.

16 Now, this is important because you can't just flip a
17 switch. I've explained this is massively parallel sequencing.
18 I've explained you can get a billion reads, but you can't just
19 flip a switch and generate a read depth of 50,000 for any
20 particular SNV, and the reason is we're looking for the needle
21 in the haystack. The ctDNA in the sample is extremely low
22 abundance. And the machines, which I haven't explained so far,
23 are not perfect. They make errors. They make errors in
24 anywhere from a percentage, 1 in 100 to maybe 1 in 500.

25 So when you actually see the sequencing data on your

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1 right-hand side on that computer screen and you see a box and
2 you see a change in the letter, most likely it's an error, not
3 actual information that came from the sample, because the error
4 rate is much higher than the actual ctDNA in the sample.

5 So what enables this is error correction. There are
6 ways that you can actually identify errors in the sequencing
7 process, remove the errors; and when you can do that, that
8 enables you to now sequence deeply into the sample to find that
9 needle in the haystack. And so that's why that claim term is
10 important because this is the way you can approach finding the
11 ctDNA that is in very minute proportions.

12 Now, I have two slides on Signatera™ and RaDaR®. So
13 in the next slide is Signatera™. And we've kind of walked
14 through these steps. There is the tumor sample that's
15 obtained. You can do whole exome sequencing. You can then
16 identify by comparing normal DNA with tumor DNA, the SNPs that
17 are associated with the cancer. You build a personalized test
18 now that involves targeted multiplex PCR, only focusing on
19 those SNPs that are tumor specific, draw blood, isolate
20 cell-free DNA, and then you can monitor the outcome of the
21 patient. This is the Signatera™ process.

22 On the last slide that I have is the RaDaR® process.
23 It also collects normal and tumor tissue. It does whole exome
24 sequencing on the normal and tumor tissue. It identifies SNVs
25 by comparing normal and tumor DNA. It develops a personalized

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1 test that focuses on those single-nucleotide variants, takes a
2 blood draw, isolates cell-free DNA, does targeted multiplex
3 PCR, and then monitors for the presence or absence of ct,
4 circulating tumor, DNA.

5 And I think that's the end of my presentation. I'm
6 happy to take other questions if Your Honor has any.

7 **THE COURT:** Thank you. We'll see what the Defendants
8 want to tell me and go from there.

9 You want to take a break before you start, or are you
10 ready to go?

11 **MS. RYAN:** Well, I do need to grab the clicker that I
12 just dropped. I believe we'll need to switch over the
13 monitors. So if Your Honor wants to take a break -- I know we
14 went for more than 30 minutes -- we can.

15 **THE COURT:** We'll go ahead. I don't want to forget
16 what I just learned.

17 **MS. RYAN:** Thank you, Your Honor.

18 As I mentioned at the beginning of the hearing, I'm
19 Liz Ryan, and I am not a doctor. So although I am going to be
20 covering some of the same concepts, I'm going to cover them in
21 the way that I learned them, which I hope will be beneficial to
22 the Court and help the Court understand the technology at issue
23 in this case.

24 The first image that Your Honor can see shows a
25 stomach tumor. The enlarged image are cells from that tumor

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1 and the blood flow, and you can see that the cells from the
2 tumor are moving into the blood, meaning that genetic material
3 from the tumor is present in the patient's bloodstream.

4 But as Your Honor heard a moment ago, there is also
5 normal genetic material present in the patient's bloodstream,
6 and cell-free DNA comes from the cells that have essentially
7 busted open in the bloodstream and allow that DNA that once was
8 inside the tumor cell to flow out into the bloodstream. And
9 for quite a long time, scientists have thought that you could
10 use that cell-free DNA, those cancer cells that are floating in
11 the patient's bloodstream, to detect and diagnose cancer.

12 And I know in Your Honor's order you had asked for
13 some prior art references. This is one. In fact, the idea of
14 using DNA to monitor or diagnose cancer goes back a very long
15 time. This is just one example, and it's from the 1990s. This
16 is a 1999 patent that references using the cell-free DNA to
17 detect cancer.

18 This is a *New England Journal of Medicine* article.
19 It's a little bit later. It's from 2013, but it references
20 back to that 1999 prior art patent.

21 So let's talk about the process of how we actually
22 use this cell-free DNA to detect and monitor cancer. The next
23 few slides are going to use some animation to explain that
24 process.

25 First, we need to determine what genetic mutations

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1 are causing the cancer. Essentially, we need to get a profile
2 of the tumor's DNA. Think of it as like obtaining a
3 fingerprint but for the cancer. To do so, we're going to
4 collect a sample of the actual tumor from the patient. We're
5 going to put the sample in a tube. We're going to do this all
6 very slowly because I have to click through it. We're going to
7 put the sample in the tube, and then we're going to put that
8 sample into the sequencer, which you heard some about, the
9 actual machine that runs the test. That sequencer is going to
10 give us a readout of the mutations that are showing up in the
11 genetic material. And those are marked here for Your Honor's
12 reference with the skull and crossbones just to hopefully
13 designate that those are the cancer mutations.

14 So how is it possible to know that those mutations
15 are associated with or caused by cancer as opposed to, as Your
16 Honor pointed out earlier, something else, some other mutation
17 in this patient's genome? Well, as Your Honor knows, DNA is a
18 code of four letters, A, C, G, and T. And knowing that, we can
19 look for mutations in principally one of three ways:

20 One, we could look at normal DNA and compare the
21 differences between normal DNA and the DNA from the sample;

22 Two, we can look at a database of known cancer
23 mutations; or

24 Three, we can look at a database of normal DNA
25 reference sequences and compare those to what we're seeing on

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1 the output from our sequencer.

2 Obtaining this sequenced data --

3 **THE COURT:** How is that different from the first
4 thing you said?

5 **MS. RYAN:** Well, the first thing is to look at the --
6 oh, look at the patient's normal DNA as opposed to looking at a
7 database of other normal DNA.

8 **THE COURT:** All right.

9 **MS. RYAN:** Obtaining those sequenced data from the
10 tumor is key. Once you have that data, there's many different
11 ways to identify the mutations from cancer.

12 And it's also important to note, Your Honor, that
13 while I've shown eight here for ease of reference and to not
14 overcomplicate the slide, keep in mind, as Your Honor has
15 probably seen in the written materials that you've read, that
16 Natera uses six. So Natera would have 16 of these lines
17 showing, and NeoGenomics would show 48.

18 We're going to keep going. And building on this
19 sequencing concept, as I've just described it, one can take the
20 tumor and sequence the DNA from that tumor to find mutations
21 that are present in the tumor. That's what we see in the top
22 in the yellow again.

23 Below, I'm showing you three of a person's
24 chromosomes. Each chromosome has a different cancer mutation
25 present throughout the chromosome. Again, they're tiny, but

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1 we've got our little red skull and crossbones again.

2 Each -- because each chromosome has different cancer
3 mutations, there are different ways that we can do the
4 sequencing to try to find them. In one example, as was just
5 mentioned, you could sequence the whole of someone's genome, in
6 other words, all their chromosomes. Alternatively, you can
7 sequence just some regions of their genome.

8 One very popular example is when you sequence just
9 the genes, which are sometimes called this exome sequencing, so
10 just a small portion that you're looking for. An exome is
11 about 1 percent of your genome. People often focus on this
12 as -- that 1 percent because much, if not all, of the important
13 information you need is present there.

14 And why would someone want to focus on only exome
15 sequencing? Because, as you might imagine, it's much more
16 expensive to sequence the entire genome than it is to sequence
17 just 1 percent.

18 Going back now to our patient, now that you've
19 identified the cancer-specific sequences, that 1 through 8 that
20 we saw in our yellow with our skull and crossbones, essentially
21 our cancer fingerprint, if you will, from the sequencing, we
22 can use to cell-free DNA analysis to see if the cancer patient
23 is coming back, the whole point of doing all of this. To do
24 so, we're going to take a sample of the patient's blood.

25 Remember, from our first slide --

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1 **THE COURT:** This is after the tumor has been
2 removed --

3 **MS. RYAN:** Yes, Your Honor.

4 **THE COURT:** -- and the radiation and the chemo, or
5 whatever the heck they --

6 **MS. RYAN:** Exactly. Exactly. Assume for purposes of
7 this presentation our patient went and had her tumor -- her
8 solid tumor removed. Maybe she needed chemo; maybe she didn't.
9 It depends on the course of treatment, and she is now going to
10 monitor to find out if her cancer is coming back following her
11 treatment.

12 So we've taken a sample of her blood. Remember, the
13 first slide, as we've shown, we've now got the patient's tumor
14 DNA in her blood. So we're going to take her blood. We're
15 going to amplify it. We're going to look for cell-free DNA.

16 (Reporter requested clarification.)

17 **MS. RYAN:** I'm sorry. I'm mindful of my 30 minutes.

18 **THE COURT:** They took longer than 30 minutes. I
19 never believe you all when you say 30 minutes.

20 **MS. RYAN:** We are terrible at estimating our own
21 time.

22 So, remember, from the first slide, the patient now
23 has the DNA in her blood from the tumor. So we're going to
24 take her blood. We're going to amplify the cell-free DNA.

25 **THE COURT:** So you hope she doesn't have it in her

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1 blood?

2 **MS. RYAN:** Well, that's what we're hoping, right. So
3 in this first sample, because we've amplified her DNA -- let me
4 run through the rest of it -- we're going to do this process
5 and put it in the sequencer. And just as Your Honor predicted,
6 she's cancer free. There's no mutation showing up after we did
7 the sample and sequenced.

8 Now, as you can see from the image, unfortunately,
9 her tumor has come back. It's pretty small right now.

10 In this slide, we're going to show the same process:
11 Blood taken, amplification, sequenced. And now because that
12 tumor is back and there is DNA floating in her blood, we can
13 see that mutation show up. We're only seeing one because the
14 tumor is not very big, and there's not a lot of that tumor
15 cell-free DNA floating through her blood.

16 Let's say she doesn't get any treatment and the tumor
17 continues to grow. We repeat the same process: Blood drawn,
18 amplification, sequencing. And now we're going to see more
19 cancer appear in the results.

20 So what is this amplification process, that middle
21 portion that I was showing in the test tube? This is an
22 example of a fragmented DNA floating in the bloodstream. It
23 was shown to you earlier as the double helix. I've shown it
24 here as a ladder because I think for our purposes the ladder is
25 just -- at least for me, it's a little simpler to understand it

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1 this way.

2 This DNA fragment could be from a tumor, or it could
3 be from a person's normal DNA. And we've got our same four
4 letters. And as you heard earlier, when you're thinking about
5 DNA, it is helpful to remember -- I learned it as the rule of
6 complementary. So I learned it a little bit different. But
7 it's this notion that C always pairs with G, and A always pairs
8 with T. They are always going to find their friends on the
9 playground. That's what these slides show. And we can see
10 them going back into the DNA fragment.

11 Now, we need to take that single fragment of DNA and
12 copy it. Copying the DNA is what allows us to produce the
13 sequence. So how are we going to do that? One cycle, the
14 amplification, refers to the doubling of DNA sequences. It
15 starts by separating two strands of DNA, and you do that by
16 heating them up. Then we add primers which are shorter strands
17 of DNA that are complementary to the DNA in the fragment that
18 we have. The primers bind to the single-stranded DNA where
19 they match, because they are always going to find their friends
20 on the playground. The primers are specific. They are
21 designed to be specific to the DNA fragment that we have taken.
22 It's called targeted or sometimes selective amplification. And
23 this shows that process.

24 Now we're going to add polymerase. That's a protein
25 that will bind a single-stranded DNA and make it double

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1 stranded by extending it with --

2 **THE COURT:** With what?

3 **MS. RYAN:** Nucleotides. The polymerase will only
4 come in at the proper place in the DNA strand. It attaches to
5 the primer, and then it adds nucleotides to the strand
6 sequentially. Once the polymerase reaches the end of the
7 fragment, we have two copies of the original DNA fragment, and
8 the cycle is complete. The process that this creates is called
9 an amplicon. That's the product of the amplification.

10 Through this amplification, we're going to keep
11 repeating the same cycle, and we can be precise. We can pick
12 where in the genome we want amplification because the
13 polymerase again will only sit down where the primers match.
14 And, remember, the primers are designed to match at particular
15 locations. We're trying to be precise. Think of it almost
16 like creating bookends. If you book-end into the primer
17 fragment, it will only grow on one side. So we have one side,
18 and then we are going to do the reverse. And by having these
19 two bookends, by the second or third replification [sic], we'll
20 end up replicating only the DNA between the bookends, only what
21 we care about. This is the process of selecting where in the
22 genome we want to copy by having multiple unique primers and
23 then replicating that time after time.

24 After two cycles, we have four copies. It grows
25 exponentially. After each cycle, the number of copies is

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1 doubled. Each amplification step comprises multiple cycles --
2 let me go back one slide -- comprises multiple cycles using the
3 same primers. So it's going to generate sufficient DNA for
4 sequencing, for plugging in our machine. We're going to keep
5 doing that, and we can do it exponentially.

6 So what is sequencing? Now that we have all of the
7 DNA copies, we're amplified. To create a quantity sufficient
8 for sequencing, we use our DNA sequencer, and we can read out
9 the results to identify the DNA sequence that is present in the
10 sample.

11 So let's now talk about what PCR is, the polymerase
12 chain reaction.

13 Suppose that you want to amplify five different
14 locus, or locations, in the genome. That's five little spots.
15 It requires five different reactions, and you can put primers
16 in five different tubes. All the different primers will have
17 different pieces of DNA. And what I have shown here is what's
18 sometimes called a fiveplex.

19 **THE COURT:** A what?

20 **MS. RYAN:** A fiveplex. You might see that in some of
21 the materials. But let's say I wanted to do six, and I have
22 six tubes. It's a sixplex. That's just where that number
23 comes from.

24 All these different primers are different pieces of
25 DNA. We're going to drop them in our tubes. And then now

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1 we've got our results ready for sequencing. We can put all
2 those tubes in our sequencing machine.

3 Targeted multiplex PCR is a different process. In
4 some settings, there are multiple targets that are of interest.
5 What I am showing you here is five separate pairs of primers
6 that I want to target in five separate regions, so five
7 separate targets. One approach would be to amplify these
8 regions in separate reactions. That means that you don't read
9 DNA from other targets. And you may want to read DNA from
10 other targets. In multiplex PCR, you're going to target all of
11 these regions in a single reaction, one tube instead of five.
12 We're going to do five things at one time.

13 Creating the primers is not simple, Your Honor. You
14 have to be unique to where you want to be in the genome. Here,
15 I'm showing five separate pairs of primers. Each pair targets
16 a different DNA sequence that's present in the reaction. These
17 sequences could be cancer specific, like the 1 to 8 on our
18 yellow card with our skull and crossbones that I showed
19 earlier.

20 In the RaDaR® process that NeoGenomics actually uses,
21 it designs primers for up to 48 regions. Remember, I said that
22 you get a printout with 48, assuming that you really get a
23 printout with --

24 **THE COURT:** I'm sorry. Assuming what?

25 **MS. RYAN:** Assuming it's really a printout and not

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1 the computer screen.

2 In multiplex PCR, you can amplify all of these
3 regions in parallel. This is an example of RaDaR® that allows
4 you to read these copies of the cell-free DNA from these
5 separate regions in one sample to determine if cancer is
6 present in this patient. So, again, we're showing them all
7 together.

8 As you can see, each of these five specific DNA
9 sequences are amplified and can now be sequenced. Because
10 we're looking for cancer mutations that are present in tiny
11 quantities, we want to increase the number of copies.
12 Essentially, more copies and searching equals more accurate
13 results.

14 Again, this notion to include many targets in the
15 same tube has been known for quite a while. This is an example
16 referencing it from 2010. It's just one example. And in 2010,
17 people knew that over 9,000 targets in a single tube could be
18 used for sequencing. It's just one example showing that.

19 Let's talk now about tagging tails and what we call
20 universal PCR, because multiplex PCR is not problem free.
21 There are reactions that could clash if you include too many in
22 a single tube at the same time, thus, the concept of universal
23 PCR.

24 For universal PCR, let's start with what tails or
25 adaptors are. These are needed for universal PCR to attach

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1 other things, such as barcodes, which I will get to in a
2 minute. I know that was referenced earlier. A common way to
3 add tails or adaptors is to ligate or attach them directly to
4 the targeted DNA such as the cell-free DNA.

5 For universal PCR, let's start with what tails or
6 adaptors are.

7 There we go.

8 We're seeing them all in our test tube. I don't know
9 if Your Honor can see that well on your slide, but you can see
10 that we've added them to the end.

11 **THE COURT:** Yes, I can see.

12 **MS. RYAN:** And now we've got the primers. So once
13 the adaptors are present on your region of interest, you can
14 use PCR primers designed to target those specific adaptors.
15 They will amplify all the DNA with these adaptors on them, and
16 they can add extra sequences, as I'm going to show Your Honor
17 in a minute.

18 Universal adaptors are added to different DNA
19 fragments, and the amplification primers are specific to the
20 universal adaptor. So all fragments within the universal
21 adaptors are amplified. The amplification step is not specific
22 to any single fragment but to the universal adaptor.

23 And that's what we're showing now. You can see the
24 primers that are attached to our adaptors, so the tails and
25 tags.

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1 As an alternative to ligating adaptors, we can use
2 PCR to make copies of cell-free DNA that contain universal
3 sequences. First, primers that have these adaptors on their
4 end bind to the cell-free DNA to make a copy of the cell-free
5 DNA, effectively amplifying it. That's what we see here.

6 We use primers with tails that don't match the DNA
7 fragment. You can see how we've got the TTT and the GGG that
8 don't match the DNA fragment to which they are attached. The
9 polymerase comes in and makes a copy such that new strands have
10 a tail. As you can see, you have a copy of the cell-free DNA
11 with the adaptor on the end. We're going to keep repeating
12 that process.

13 In the next PCR cycle, a copy of the cell-free DNA
14 binds to a second primer and makes a copy of a copy. This
15 creates new molecules with a tail on them, which we can use to
16 introduce a new DNA sequence. In every PCR cycle, you generate
17 tagged products that are the same, meaning that the tagged
18 products from the tenth cycle are no different than the tagged
19 products from the fifth cycle. And that's what this shows.
20 We're going to keep doing it.

21 This concept of tagging is also something that's been
22 known for a while. Here is an example of prior art that
23 references it from 2010. It's just one example of that.

24 Your Honor's heard some about barcoding, so I wanted
25 to go through that briefly as well. DNA barcodes can be used

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1 to identify which sample a DNA sequence comes from. In RaDaR®,
2 which is our product, after we've copied the cell-free DNA with
3 primers that include universal sequences, NeoGenomics then does
4 that universal PCR process that we just walked through adding
5 barcodes.

6 So in this example, say that we have three different
7 patients, and we want to sequence DNA from three different
8 patients all at the same time. And you're probably asking why
9 would we want to sequence DNA from three different patients all
10 at the same time? And it goes back to what I mentioned
11 earlier, that it is more cost-effective to do so.

12 Ordinarily, putting multiple patients together in a
13 single tube would, as you might expect, be problematic because
14 how would you know which DNA came from which patient. The
15 sequencer would simply mix it all together. But when we do an
16 amplification with a tail, we end up with a different tail for
17 each product -- we end up with a different tail for each
18 patient -- not product, patient -- meaning a different ACGT
19 combination for each patient. That's what you're looking at in
20 this next slide. And now we're going to put them in our tubes.
21 It's like putting -- hence, the name -- a barcode on the DNA
22 that you link to a specific patient.

23 Now that we have the barcodes for each patient, we
24 can mix all three patient samples together. We can put them on
25 our sequencer, and we can tell which DNA came from which

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1 patient.

2 **THE COURT:** You can tell what?

3 **MS. RYAN:** Which DNA came from which patient.

4 Barcoding concept, also not new. It's been known
5 since at least 2010. This is one example of that.

6 Also, for the sake of confusion, I've highlighted 454
7 Barcode Primers. That's not a patent number, Your Honor. That
8 is the name of a sequencer, the machine. The platform that
9 used to exist, it no longer does. But it's the same thing as
10 the machines that we use now.

11 Finally, Your Honor, I'd like to cover a concept
12 called sequencing depth. Let's assume we have DNA from someone
13 with cancer. As we said, there are known cancer mutations on
14 different regions of the chromosome. Now, if you don't
15 sequence very much of the DNA, you could miss detecting the
16 tumor in the patient, but because --

17 **THE COURT:** I'm sorry. Hold on just one second.

18 (Pause in the proceedings.)

19 **THE COURT:** Can you back up? I'm sorry.

20 **MS. RYAN:** Yes, Your Honor.

21 So sequencing depth is the last concept I would like
22 to cover.

23 So, again, Your Honor, we're going to assume that we
24 have DNA from someone with cancer. There are known cancer
25 mutations on different regions of human chromosomes. If you

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1 don't sequence very much of the patient's DNA, you could miss
2 detecting the tumor from the patient. But because most of the
3 DNA on a chromosome comes from normal human genomes, not
4 cancer -- you can see I've got little bitty cancers. Because
5 most of the DNA comes from the normal human genome and not
6 those cancer mutations, what we want is to sequence over and
7 over to find as many copies of the DNA from various known
8 cancer mutation regions on the chromosome.

9 Sequencing depth, this last concept, refers to the
10 number of times you sequence a region on the chromosome. A
11 higher sequencing depth means a higher sensitivity, a better
12 read on the sequence, which is helpful in detecting mutations
13 in the cell-free DNA. And that's what this image is showing.
14 We're putting all this into our sequencer over and over because
15 we're looking for those mutations on the chromosome, and we
16 found one.

17 As with the other concepts we have discussed today,
18 Your Honor, sequencing depth has been known for a long time.
19 This is just one example that references it from 2010. And
20 this is another example from 2012.

21 That completes my presentation, Your Honor.

22 **THE COURT:** All right. Thank you.

23 Why don't we take a short break. We'll come back.
24 If you all want to take five minutes for anything else you want
25 to add in light of that and then I will give you a few more

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